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Comparison of serological techniques for the diagnosis of leptospirosis.

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COMPARISON OF SEROLOGICAL TECHNIQUES FOR THE DIAGNOSIS OF LEPTOSPIROSIS

by

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INTRODUCTION

The diagnosis of an infectious disease such as leptospirosis may be established at different levels of certainty. The presence of signs suggestive of leptospirosis in a group of animals will lead the observer to make a presumptive diagnosis which may be confirmed by the isolation of the causative agent. However, the leptospirae are not recovered from an infected animal with any degree of facility either by animal inoculation or cultivation on suitable media. The effort and expense involved in establishing a diagnosis by recovering the organism make it necessary to accept methods which involve a lower degree of certitude. The detection of antibodies against leptospirae in the serum, milk or urine of animals falls into this category. The serological confirmation of a diagnosis of leptospirosis is less accurate since antibody titers are measured by arbitrary techniques which vary in replication in the hands of different workers and possess intrinsic sources of error.

At the start of this project many laboratories throughout the country were engaged in examining sera for antibodies with a variety of serological tests. It was felt that it would be of value to develop a comprehensive survey of these techniques by submitting to each of the laboratories an identical set of test sera for their examination. The laboratories were requested to complete a questionnaire which elucidated the important details of the procedure employed or furnish a description or reference concerning it. The mass of data obtained was carefully evaluated by statistical analysis to gain basic information about the diagnosis of leptospirosis by antigen-antibody reactions. Knowledge

about the methods employed might lead to the establishment of a single universal standard technique which would replace the many competing tests now in existence. If some of the laboratories were permitting innovations and inaccuracies then the survey would reveal the effect of these discrepancies. The study would also show the natural experimental error of the techniques most commonly used and furnish a base line for the future evaluation of efforts directed toward improved standardization.

The livestock industry is vitally concerned with this disease because of its costly ravages due to loss from abortion, drop in milk yield, and mortality. Past experience with the control of brucellosis by means of an excellent serological tool, the tube or plate agglutination test, creates a demand for a similar approach to the problem of leptospirosis. Farmers whose herds are free of leptospirosis ask that the veterinary profession use serological means to detect infected animals which may be spreaders of virulent leptospirae and set up regulations to quarantine or control the movement of such livestock. The development of standard serological techniques for the serodiagnosis of leptospirosis is a necessity. This study reveals some of the sources of variation in the procedures employed and correlates aberrant test results with laboratories guilty of deviations from described protocols. The analysis of variance is used as a new method for evaluating and comparing serological data. This survey which was performed so that an accurate picture might be obtained of the problem demonstrates the need for the adoption of standardized methods for the serodiagnosis of leptospirosis.

After the examination of the data an urgent need was felt for controlled experiments to confirm some of the conclusions obtained from the survey. This situation applied particularly to the agglutination-lysis test in which many sources of variation were present. Since the survey data were influenced by many associated factors which were lacking in the controlled experiments the conclusions reported in this part of the thesis may be more valid.

Consideration of the value of the Brucella milk ring test in locating herds of dairy cattle infected with brucellosis encouraged the investigation of how the existing serological techniques for the diagnosis of leptospirosis might be adapted for use on composite herd milk samples. The last part of the thesis describes the detection of antibody levels in milk samples obtained from infected herds. The whey titers of these milk specimens are compared with the serum titers obtained on the individual cattle in these herds using the agglutination-lysis and Stoenner plate techniques. Any program designed to control or eradicate leptospirosis in an area populated predominantly by dairy cattle herds would find this screening test an economical adjunct.

The importance of infection with Leptospira pomona in this country as compared with infections involving other serotypes such as L. icterohaemorrhagiae and L. canicola has limited the scope of the survey and related experimental work to this serotype. The antigen-antibody reactions evaluated in this thesis involve only those produced by Leptospira pomona.

PART I. SURVEY AND EVALUATION OF SEROLOGICAL TECHNIQUES AS EMPLOYED ON A NATIONAL SCALE

LITERATURE REVIEW

The comparison of serological procedures of different laboratories by means of the simultaneous examination of aliquots of the same set of sera has been a routine procedure with organizations connected with public health and livestock sanitation. Most of this work has been carried out with a reasonable desire for secrecy and often was performed for laboratories which were part of a larger organization. There were no published records of these surveys available.

In 1948, Griggs and Case (1) reported significant variations in Brucella agglutination reactions on the same blood specimens in different laboratories. A total of 215 human sera was examined by four different laboratories. Sera reported positive by one laboratory and negative by another were 64 in number or 30 percent of the group. It was felt that some of the antigens employed were so insensitive as to fail to produce positive reactions with sera from proven cases of brucellosis. The use of an antigen prepared from several strains of Brucella abortus was recommended.

Borg-Petersen and Fagraeus (2) in 1949 described the effects of antigen density and other factors on the serum titer using the agglutination-lysis test for leptospirosis. Having observed considerable discrepancy between the results of tests performed on the same sera by two Swedish laboratories they systematically studied the relation between antigen density and serum titer. Methods used by other laboratories to standardize the antigen were reviewed and it was concluded that marked variations existed in the density of the antigen

used by these laboratories. Precise experiments were carried out which related a twofold increase in the titer of a serum to a fourfold dilution of the antigen culture. Concurrently, variation in the titer of a serum was related to the use of different strains of the same leptospiral serotype. Increasing age of the antigen culture decreased its sensitivity in the agglutination-lysis test. No effect was attributed to differences in the batches of media used to grow the antigen culture.

Recommendations were given to attempt to standardize the test in regard to the factors which were found to be sources of variation. The advantages of a standard technique were pointed out in some detail. However, the variation due to different end point determinations and dilution schemes was accorded the small attention of a footnote commentary. It would appear that more precise information would have been gained by a better experimental design which included more sera and an adequate statistical treatment. The twofold difference in serum titer observed should have been compared to the standard deviation expressed as a dilution factor for the entire experiment. The standard deviation may have actually been a twofold factor.

With the development of serological tests employing extracts and killed suspensions of leptospira as antigens it was necessary that these techniques be correlated with the agglutination-lysis test. These procedures were more desirable than the agglutination-lysis test from the aspects of safety and simplicity. An evaluation study was conducted in 1956 by Gleiser and Alexander (3) at the Walter Reed Army Institute of Research to determine how well some of the newer techniques correlated with the agglutination-lysis test as conducted at their laboratory (4).

A total of 86 sera were examined with the following six test procedures: agglutination-lysis test, macroscopic agglutination test, plate-capillary tube agglutination test, complement-fixation test with sonic-vibrated antigens, erythrocyte sensitizing test, and the hemolytic test. The tests were employed in the laboratories where they were originally described and the sera were coded. The titers obtained on the sera were classified arbitrarily as "positive" or "negative" and the correlation studies were performed on these simplified data. The plate-capillary tube test correlated most closely with the agglutination-lysis test. Good correlation was obtained with the macroscopic agglutination test but the complement-fixation test failed to detect some of the positive sera. The erythrocyte sensitizing test and the hemolytic test correlated well on human sera but showed only 58 percent agreement on the animal sera. A thorough evaluation of the merits of these tests as routine diagnostic procedures was included in their report. However, since the techniques were applied by the laboratories developing them a question might be asked as to how well the techniques would have compared in the hands of other workers.

Serological titers by their nature have not readily lent themselves to direct mathematical treatments. The titers may be reduced to positive or negative classifications as in the above report. However, much information was lost in this process. Perkins (5) suggested gaining more useful data through the calculation of geometric mean antibody titers for groups of sera by transforming the titer values into logarithms. This method was adopted for handling the serological data evaluated in this thesis and was of considerable assistance.

In 1957 a committee was appointed by the American Association of Veterinary Bacteriologists to study the methods employed for the serodiagnosis of leptospirosis in the various veterinary diagnostic laboratories. At the meeting of the association in 1958 a complete report was presented by the committee (6). Part I of the report listed the sixty-three laboratories participating in the survey and Part II presented a tabulation of the results from a questionnaire completed by these laboratories. There were six different testing procedures reported: plate, agglutination-lysis, capillary tube, A-L with formolized antigen, complement-fixation, hemagglutination with L. biflexa, and microscopic slide agglutination. A diversity of dilution schemes, end point determinations, titer interpretations and other factors was revealed. Many laboratories using methods adequately described in the literature had introduced innovations into their techniques. The need for an evaluation program was clearly expressed. It was suggested that a central agency should supply either check sera or standardized antigen for the use of the diagnostic laboratories. The questionnaire survey provided some interesting information concerning the variegated patterns of leptospirosis serology. However, it did not indicate what significance might be attached to some of the variations in the techniques most commonly employed such as the agglutination-lysis and plate agglutination tests.

METHODS AND MATERIALS

As a necessary preliminary to the serum survey a letter was sent to all laboratories engaged in the serological diagnosis of leptospirosis announcing the project and requesting a return letter if the laboratory wished to participate. Public health laboratories engaged in examining human sera as well as veterinary research and diagnostic laboratories were contacted. It was felt that the problem was a mutual one and the larger the number examining the sera with a particular serological technique the more valid would be the conclusions drawn concerning it. Each laboratory was designated by a code number so that the identity of the laboratory would not be revealed. It was recognized that a natural desire to avoid embarrassment might discourage some of the laboratories from entering the survey. The code enabled each laboratory to view its own readings in comparison with those of all of the others without risking possible criticism. This scheme increased participation and was considered an essential part of the survey.

It was decided to distribute a total of twenty-five sera. Since the efficiency of the survey increased as the square root of the number of samples, twenty-five was considered a minimum number. On the other hand a larger number might have discouraged some of the laboratories from completing the tests. It was estimated that about four hours of laboratory time would be required to process the samples. One negative serum was included as a check on antigen sensitivity. More negative samples might have been appropriate. However, the assumption was made that the least that could be asked of any serological process would be

the recognition of the absence of antibody. This supposition was not justified when the results were tabulated.

The sera were selected so that a variety of titers from low to high were offered for examination. The sera were obtained from the following sources:

Sera numbered 1-8, and 11 were composite samples prepared by mixing L. pomona positive, bovine, sera obtained from Dr. P. C. Bennett, Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa.

Serum number 9, possessing an extremely high antibody titer, was obtained from Dr. K. J. McMahon, Department of Bacteriology, Kansas State University, Manhattan, Kansas. A post-mortem diagnosis of chronic interstitial nephritis was made on the cow from which this serum was collected.

Serum number 10 was obtained from a healthy cow in the experimental herd at the College of Veterinary Medicine, Iowa State University, Ames, Iowa and did not contain antibody against L. pomona.

Sera numbered 12-15 were obtained from Dr. R. W. Gillespie, Department of Veterinary Microbiology, Washington State University, Pullman, Washington:

Serum number 12 - Sample was taken from a cow experimentally infected on 1/1/59 and bled about 3/18/59. The animal shed leptospirae in the urine.

Serum number 13 - Sample was taken from a cow experimentally infected about 2/1/59 and bled about 3/18/59.

The animal shed leptospirae in the urine.

Serum number 14 - Sample was taken in March from a cow naturally infected in late November or early December. L. pomona was isolated from this animal in December and January.

Serum number 15 - The history of this sample was the same as serum number 14.

Sera numbered 16-22 were furnished by Dr. R. L. Morter, Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan, who identified the sera as follows:

Sera numbered 16 and 17 - Samples were obtained from sheep experimentally infected with L. pomona Wickard.

Sera numbered 18-21 - Samples were obtained from cattle experimentally infected with L. pomona Wickard.

Serum number 22 - Sample was obtained from a pig experimentally infected with L. pomona Ohio.

Sera numbered 23-25 were supplied by Dr. Erskine V. Morse, Veterinary Medical Research Institute, Ames, Iowa, who identified the sera as coming from swine experimentally infected with L. pomona Ohio.

The sera were filter sterilized with a Seitz filter and dispensed into one dram, screw-top vials. All sera were maintained in a frozen state until shipment to the laboratories. The sets were packaged in cardboard, blood sample boxes and all mailed at the same time. A set of blank test charts and an appropriate questionnaire were enclosed in each box. Laboratories employing the agglutination-lysis test received

a questionnaire which, if properly completed, would indicate all of the essential details of this multiform test. The questionnaire sent to the laboratories performing either of the Stoenner tests was planned so as to detect essential deviations from the protocol as described by Stoenner. Mimeographed samples of these two forms are presented in the Appendix. Laboratories using one of the more unusual techniques such as complement-fixation were requested to describe their procedure or furnish a citation.

Upon the return of the results from each laboratory a careful study was made to determine whether the stated titers in the test chart were the same as those achieved by the dilution protocol described in the questionnaire. All readings for the agglutination-lysis test were corrected to 50 percent end points at the final dilution achieved after the addition of antigen to the serum dilution. Titer values were converted to logarithms of two decimal places to the base 10. A geometric mean titer was calculated for each serum by adding the logarithms, dividing the total by the number of laboratories, and converting the quotient to the antilogarithm. Negative readings were assigned a positive titer of 1-2 which was considered a reasonable, non-specific level of antibody. The logarithmic value, 0.30, was, therefore, used for negative readings in calculating the mean titer. A special modification was used for the agglutination-lysis test because of the diversity of dilution schemes utilized. Each titer value was corrected to a logarithmic midpoint between the 50 percent end point titer and the next higher dilution titer in the scheme.

Some laboratories did not determine end point titers on all of

the sera, although this had been requested. Two laboratories were discarded from the statistical analysis for this reason. Some of the sera were broken in transit. If no more than five serum titers were missing or not determined to end point in the results of a laboratory, a correction technique was applied. The geometric mean titer of the sera determined to end points was determined and compared with the geometric mean titer of a suitable laboratory reporting on all twenty-five sera. The difference between the two means was used to calculate the missing titer values. In the statistical analysis the total number of substituted or missing values was subtracted from the number of degrees of freedom used to calculate the residual variance.

In order to facilitate the calculations involved in the statistical analysis the data for the five most frequently reported techniques were coded on punch cards and processed through an International Business Machine 650 Computer.

RESULTS AND EVALUATIONS

General Review

Seventy-three laboratories participated in the survey with some performing more than one type of procedure. Approximately 85 percent coverage of laboratories employing serological tests for the diagnosis of leptospirosis in the country was attained. The Stoenner test which was actually two different techniques using the same antigen enjoyed the greatest popularity. There were thirty-six laboratories utilizing the Stoenner Plate test (7) and eight laboratories reporting the Stoenner Capillary tube test (8) making a total of forty-four laboratories. The antigen for these two tests was commercially available and the techniques were well described initially in the literature. The support of the commercial houses undoubtedly contributed to the widespread adoption of these techniques. Veterinary diagnostic laboratories predominated in this group. The agglutination-lysis test (9) was employed by twenty-nine laboratories of which seventeen were engaged primarily in research and twelve were performing diagnostic services. This technique which employs the living culture as antigen was the first method used to detect leptospiral antibody and has remained the choice of the research laboratory. The Galton test (10) was utilized by six laboratories and was conducted with a killed suspension of leptospirae. This test originated in a public health laboratory and was reported only by establishments engaged in public health work. Three public health laboratories reported the use of the microscopic agglutination test with formalin-treated antigen. The complement-fixation test was

reported by two public health laboratories. However, only one laboratory cited a reference (11). Four laboratories reported serological techniques developed and utilized on an experimental basis only.

Experimental Tests

Four techniques were reported which were employed only by the laboratories reporting them. The titers of the test sera for these experimental procedures are presented in Table 1.

Table 1. Experimental tests

Serum number	Laboratory number			
	69	74	66	77
1	160	1,000	400	100
2	160	1,000	800	400
3	160	1,000	800	100
4	40	1,000	800	10
5	160	1,000	1,600	100
6	10	10	100	N
7	40	1,000	400	40
8	10	100	100	N
9	640	10,000	51,200	10,000
10	N	N	100	N
11	160	10,000	800	100
12	40	1,000	400	N
13	40	1,000	100	10
14	640	10,000	6,400	100
15	640	10,000	51,200	10,000
16	40	1,000	1,600	400
17	40	100	400	N
18	40	100	100	10
19	10	10	100	N
20	40	100	200	10
21	160	1,000	400	100
22	10	N	N	N
23	10	100	100	N
24	160	10,000	200	40
25	40	1,000	200	400

In all of the tables the serum titers will be reported as the reciprocals of the 50 percent end point titers. Negative test readings will be designated by the capital letter "N".

Laboratory 69 examined the sera with a technic developed by combining parts of the Stoenner and Galton tests. A protocol accompanied the test chart:

> "Antigen is prepared in our laboratory from L. pomona S-91 according to Galton. Antigenicity and specificity are determined against hypersera from at least ten serotypes before releasing for use. Density is standardized to 30% transmission on a B & L spectronic 20 at 550 mu."
>
> "Serums are diluted so when 0.04 of each dilution is mixed with 0.04 of antigen the final test dilutions are 10, 40, 160, and 640. The serum-antigen mixture is rotated 150 rpm for five minutes at room temperature (25°C). At the end of this period, they are slowly rotated by hand and read. Any unmistakable agglutination is recorded as positive."

The titers reported by laboratory 74 were determined by observing the macroscopic agglutination of a killed suspension of leptospirae in small conical glass tubes (12). These readings may be supplemented by microscopic observation of the supernatant fluid, if necessary.

The test used by laboratory 66 was quite unusual in that the antigen consisted of latex spheres mixed with a killed suspension of leptospirae (13). The test designated a positive titer in the 1 : 100 dilution for the negative serum, number 10. Serum number 22 which possessed a low level of antibody was classified as negative.

The test reported by laboratory 77 employed a genus-specific antigen prepared by the alcoholic extraction of a saprophytic leptospiral serotype, L. biflexa (14,15,16). This antigen was described as causing hemolysis of sheep erythrocytes in the presence of antibody and complement.

Serum numbers 6, 8, 12, 17, 19, and 22 which contained low to medium levels of antibody were classified as negative. The lower sensitivity of this technique for animal sera has been reported previously (3).

Complement-fixation Test

This test was performed on the test sera by two laboratories each using a different protocol. The results are presented in Table 2.

Table 2. Complement-fixation test

Serum number	Laboratory number	
	66	65[a]
1	50	64
2	70	64
3	70	64
4	30	64
5	125	64
6	10	16
7	50	64
8	10	16
9	1,000	64
10	20	N
11	125	64
12	40	64
13	25	64
14	300	64
15	1,300	64
16	60	64
17	15	16
18	15	16
19	N	4
20	20	64
21	30	64
22	N	4
23	N	N
24	15	64
25	15	16

[a]End point titers not determined beyond the 1-64 dilution.

Since laboratory 65 did not carry end point determinations beyond the 1-64 dilution, it was impossible to compare the procedures.

Some complement-fixing antigens are genus-specific permitting the use of this technique to detect infections with any serotype. However, the arduous task of standardizing reagents will serve to make the complement-fixation test unsuitable for the diagnosis of leptospirosis.

Microscopic Agglutination Test with Formalin-treated Antigen

No reference was given for the performance of this test since each laboratory using it had developed their own technique. Basically, the test was performed by mixing a formalin-treated antigen with the serum dilutions and observing the agglutination of the leptospira with a microscope. The serum titers reported are presented in Table 3.

Casual observation indicated considerable variation among the values obtained by these laboratories. This was brought out in Table 4 where the data were processed by the statistical treatment, analysis of variance (17).

The variation among the titers of the sera was an essential part of the experimental design and was of no interest. However, the F ratio for laboratories was calculated to be 46.66 compared to a theoretical F of 5.08 with a probability of 0.01. An F value of this magnitude would seem to indicate the existence of three different testing procedures. A comparison among the three means was made using the Q test (17) to determine where significant differences existed at the 5 percent level. In Table 5 the three laboratory means are seen to be significantly different from one another since the actual

Table 3. Microscopic agglutination test with formalin-treated antigen

Serum number	Laboratory number			Geometric mean titer
	76	64	68	
1	160	256	1,024	350
2	1,280	2,048	8,192	2,800
3	640	1,024	8,192	1,740
4	160	512	4,096	690
5	640	10,192	8,192	3,800
6	20	64	512	186
7	160	1,024	2,048	690
8	20	128	512	110
9	20,480	81,536	13,172	60,000
10	N	16	N	4
11	1,280	2,048	4,096	2,200
12	320	512	1,024	550
13	80	512	512	280
14	2,560	20,384	8,192	7,600
15	81,920	163,073	32,768	76,000
16	80	512	2,048	440
17	80	256	1,024	280
18	80	128	256	138
19	10	32	256	44
20	160	256	1,024	350
21	160	512	4,096	690
22	40	128	64	69
23	40	128	128	87
24	1,280	5,096	8,192	3,800
25	160	1,024	4,096	870

differences are larger than the theoretical differences which are enclosed in the parentheses.

The advantage of using a killed antigen in this test was offset by a striking degree of variation between laboratories. The standard deviation for this technique expressed as dilution factor was 1:2.10 or about a twofold factor.

Table 4. Analysis of variance of microscopic agglutination test with formalin-treated antigen

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera	24	64.6821[a]	2.6951
Laboratories	2	9.6459	4.8230
Residual	48	4.9615	0.1034

[a]All calculations performed after transformation of the titer values to logarithms to the base 10.

Table 5. Comparison among laboratory means, microscopic agglutination test with formalin-treated antigen

Laboratory number	$\bar{X}$	$\bar{X}$-2.46	$\bar{X}$-3.05
68	3.32	0.86 (0.22)[a]	0.27 (0.18)
64	3.05	0.59 (0.18)	
76	2.46		

[a]Difference between means at the 5 percent level.

Galton Test

The Galton test (10) was described as a rapid macroscopic-slide test using a formalin-killed suspension of leptospiral cells. A drop of antigen was mixed with a measured amount of the serum dilution on a glass plate. This plate was mounted on a mechanical rotator and oscillated for four minutes at 125 revolutions per minute. The drops were then examined macroscopically over a light box for agglutination.

The titer results of the six laboratories using this test compared with the geometric mean titer for each serum are shown in Table 6.

Table 6. Galton test

Serum number	Laboratory number						Geometric mean titer
	57[a]	61	75	58	56	65	
1	32.5	119	188	60	32.5	11.9	50
2	60	119	325	60	60	60	89
3	32.5	60	325	60	32.5	18.8	54
4	18.8	60	60	32.5	18.8	18.8	30
5	93	188	600	188	32.5	60	126
6	32.5	18.8	11.9	N	6.5	6.5	9.1
7	162	119	60	60	32.5	188	87
8	11.9	11.9	N	N	6.5	N	4.4
9	20,312	6,000	600[b]	3,250	60[b]	N	7,400
10	18.8	18.8	N	N	N	N	4.2
11	93	188	325	188	60	60	126
12	32.5	32.5	32.5	18.8	18.8	18.8	25
13	60	32.5	32.5	18.8	60	18.8	33
14	468	600	600	325	60[b]	60[b]	480
15	7,421	6,000	600[b]	3,250	60[b]	60[b]	5,200
16	93	60	60	60	32.5	32.5	52
17	93	32.5	60	60	18.8	18.8	40
18	60	32.5	60	60	11.9	18.8	34
19	N	18.8	11.9	N	N	N	3.9
20	32.5	32.5	60	60	32.5	32.5	40
21	162	60	325	60	32.5	60	85
22	N	6.5	N	N	6.5	6.5	3.6
23	18.8	11.9	N	N	6.5	6.5	5.8
24	297	119	325	119	32.5	60	118
25	60	32.5	325	60	18.8	60	59

[a]This laboratory did not report titer values according to the dilution scheme described by Galton.

[b]These titers were not end point determinations and were not used in calculating the geometric mean titer.

Interesting features of this test were the low titers determined on the sera and the irregular dilution scheme. However, the dilution protocol was carefully described and strict adherence to it may have produced better replication among the laboratories as seen in the analysis of variance (Table 7).

Table 7. Analysis of variance of the Galton test

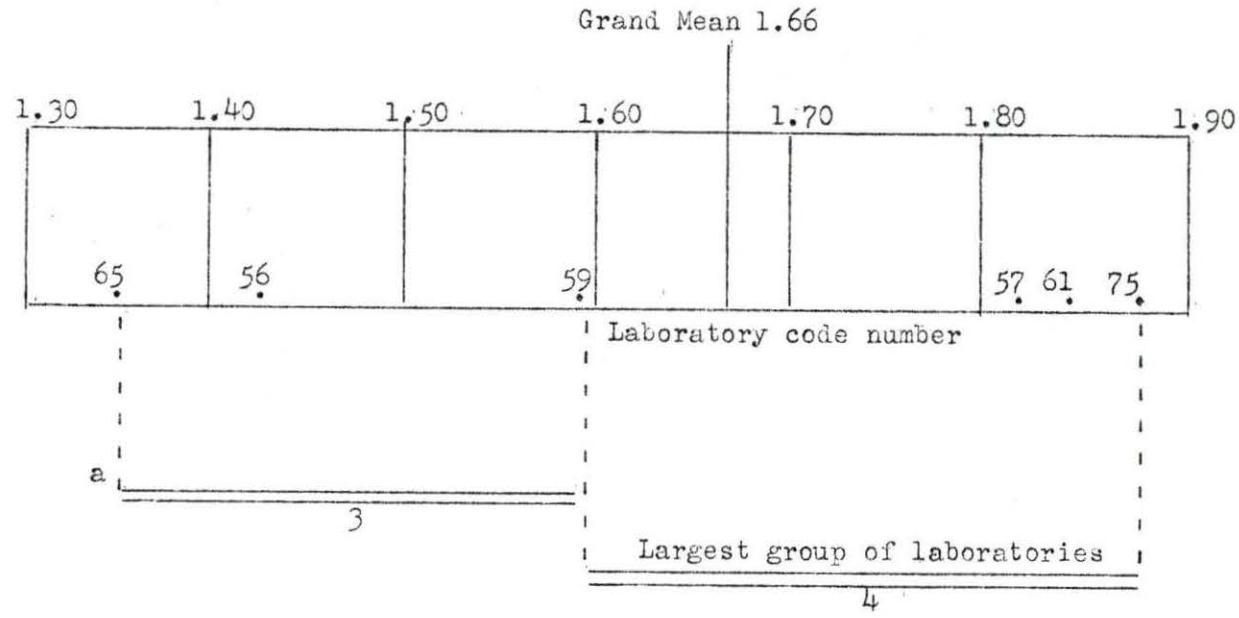
Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera	24	77.2645	3.2194
Laboratories	5	7.0620	1.4124
Residual	113[a]	18.3520	0.1624

[a]Seven degrees of freedom subtracted for missing data estimations.

An F ratio for laboratories was calculated to be 8.70 compared with an F of 3.17 having a probability of 0.01. This was the lowest degree of systematic variation among laboratories encountered in the survey. It was considered to be due to the standard dilution protocol used which actually specified the size of pipet and volumes of diluent. The possibility also existed that these laboratories had developed better training programs and had more competent personnel. The standard deviation calculated from the residual variance was found to be a dilution factor of 1:2.53.

In Figure 1 the laboratory means were arranged on a linear scale with the mean values and code numbers indicated. These laboratories whose means were not significantly different from one another at the

Figure 1. Laboratory means of the Galton test arranged on a linear scale.



[a]Each bar connects laboratory means not significantly different from one another at 5% level.

5 percent level were joined by the double lines seen in the lower part of the figure. The distribution was slightly unbalanced with the largest group of laboratories not significantly different from one another located above the grand mean. Laboratory 65 was consistently low on all sera and recorded as negative a serum with a high titer.

Stoenner Capillary Tube Test

The capillary tube test (8) was the original technique developed by Stoenner and the plate test (7) was a subsequent modification. The tubes were filled by capillary action with equal amounts of serum dilution and a killed antigen and mounted in a slanting position on a plasticine block. After incubation at 37°C. overnight the tubes were placed in a refrigerator at 4°C. for an hour. The tubes were then read by observing the clumps of agglutinated leptospirae clinging to the inside surfaces of the tubes. Final serum dilutions used in this test were fourfold dilutions starting with a 1-10 dilution. A time-saving feature of the test was the screening technique utilized in which a wire loop of serum, 0.005 ml, was mixed with 0.03 ml of antigen on a glass plate. After stirring and incubation at room temperature, the drops of serum and antigen were examined for agglutination. A positive serum was then diluted to determine the end point titer and the negative serum was discarded. Some sera with low titers of antibody reacted to the plate screen test which was actually a 1-7 dilution, but did not react to the 1-10 dilution. These serum titers were designated by "P" for positive in the tables. The capillary tube test was performed on the test sera by eight laboratories. The results are presented in Table 8.

Table 8. Stoenner capillary tube test

Serum number	Laboratory number								Geometric mean titer
	44	37	51	80	36	43	17	29	
1	640	640	2,500	160	P[a]	160	40	160	180
2	2,560	640	12,500	640	10	640	160	640	550
3	640	40	500	160	10	160	40	160	110
4	160	40	500	160	P	40	10	40	52
5	2,560	160	2,500	160	10	160	10	640	190
6	N	10	20	10	N	10	10	10	7.2
7	160	40	500	160	P	160	40	160	87
8	N	10	N	N	N	N	N	P	2.9
9	40,960	10,240	62,500	40,960	640	10,240	163,840	10,240	18,000
10	N	N	20	N	P	P	N	N	3.6
11	160	160	2,500	640	10	10,240	160	640	380
12	640	10	500	40	P	160	10	40	52
13	10	10	500	40	P	40	40	40	31
14	2,560	2,560	62,500	640	160	10,240	640	2,560	2,300
15	40,960	2,560	62,500	40,960	2,560	10,240	2,560	10,240	11,000
16	640	640	2,500	160	10	2,560	160	640	380
17	160	40	500	160	P	160	40	160	87
18	160	40	500	40	10	160	40	40	65
19	P	N	N	N	N	N	N	N	2.3
20	640	40	500	160	P	40	40	160	87
21	640	640	2,500	160	10	640	40	160	220
22	10	N	N	N	N	40	N	10	4.4
23	10	10	N	40	P	10	10	10	9.3
24	2,560	160	2,500	640	10	10,240	160	640	540
25	640	160	2,500	160	10	160	40	160	160

[a]Positive reaction on the screening test at a 1-7 dilution, negative at the 1-10 dilution.

Analysis of variance was performed on the data and a large component of variance was associated with variation among laboratories (Table 9).

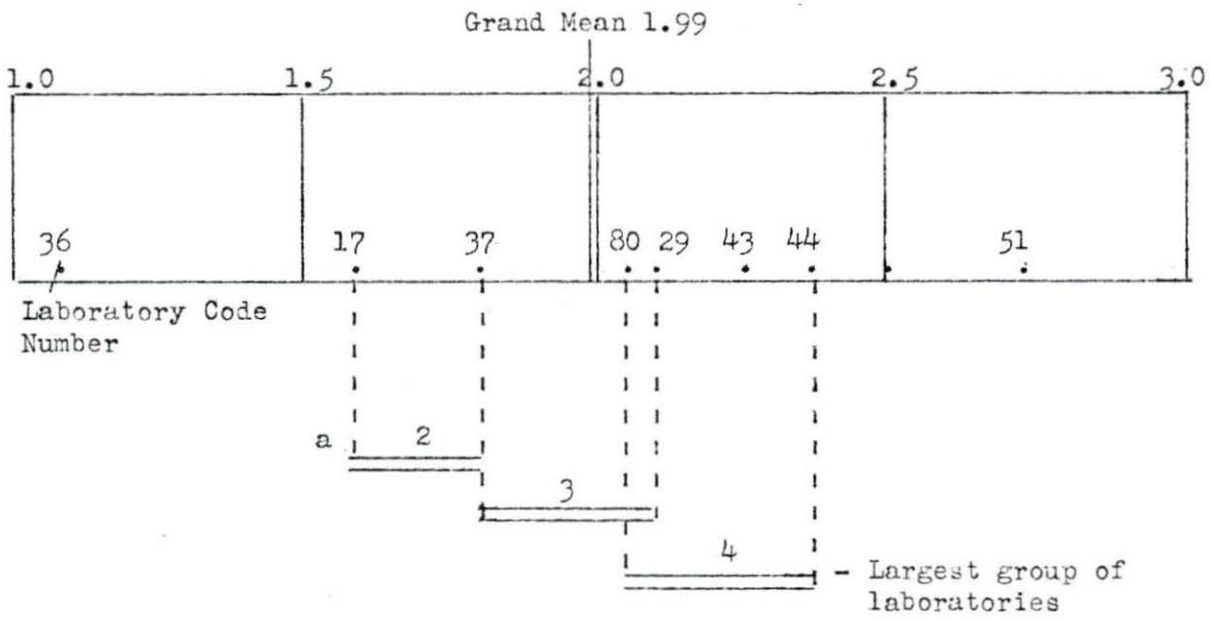
Table 9. Analysis of variance of the Stoenner capillary tube test

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera	24	199.5938	8.3164
Laboratories	7	43.7028	6.2432
Residual	168	33.8717	0.2016

For laboratories the F ratio was 30.96, compared to an F value of 2.75 at the one percent level. The laboratory means were compared and the questionnaires examined for deviations from the standard protocol described by Stoenner. The result of the application of the Q test to the means is presented in Figure 2.

It may be observed from Figure 2 that two of the laboratory means were significantly different from all of the others. The largest group of laboratory means not differing from one another at the 5 percent level were located above the grand mean. Laboratory 51 employed a different dilution scheme than the one described by Stoenner and was at the high end of the scale. Laboratory 36 determined end point titers by reading 100 percent agglutination instead of the correct method in which the end titer would be the highest dilution showing visible agglutination. Such a practice would consistently lower the titer determinations made by this laboratory. The standard deviation of this technique was dilution factor of 1:2.81.

Figure 2. Laboratory means of the Stoenner capillary tube test arranged on a linear scale.



[a]Each bar connects laboratory means not significantly different from one another at 5% level.

Stoenner Plate Test

It was of great interest to study this most universally employed serological technique. The Stoenner plate test had the advantage of becoming available when the need for a simple, safe, and rapid procedure was most acute. This test became firmly entrenched and it may be difficult to supplant if a superior method should be developed. The titers reported by the laboratories are presented over several pages in Table 10. For convenience, the geometric mean titers for each serum are repeated on each page of the table.

The protocol of this test was similar to the capillary tube technique previously described except in the determination of end point titers. The serum dilution was mixed with an equal volume of antigen on a glass plate, incubated for six minutes, rotated by hand for one minute, and examined macroscopically for agglutination. It was expected that the clear sterile test sera in the hands of laboratories routinely performing this test would result in excellent replication of titer readings among laboratories. Unfortunately, casual observation of Table 10 did not confirm this opinion. The analysis of variance is presented in Table 11.

An F value of 20.18 was calculated for laboratories compared to the theoretical F at the one percent level of 1.71. This tremendous systematic variation among laboratories compared unfavorably with an F ratio of 3.22 ($F = 3.51$, $P_{.01}$) calculated for five repeat runs made on the same set of test sera by the author. The F ratio for laboratory variation on the Galton test was only 8.70 with a much smaller number of laboratories. Some of the variation among laboratories on the

Table 10. Stoenner plate test

Serum number	Laboratory number						Geometric mean titer
	24	41	15	52	26	27	
1	40	80	160	160	160	160	93
2	160	320	640	160	160	160	210
3	40	40	160	160	160	160	91
4	40	40	160	160	40	40	55
5	160	160	160	640	640	160	260
6	10	P[a]	10	10	10	10	7.2
7	40	40	160	160	160	40	85
8	N	P	10	N	10	10	4.5
9	2,560	2,560	2,560	10,240	10,240	10,240	3,700
10	N	P	N	N	40	P	3.8
11	160	160	160	160	640	640	250
12	40	40	40	40	40	40	54
13	40	40	10	40	40	40	31
14	640	640	640	2,560	2,560	2,560	1,100
15	2,560	2,560	2,560	10,240	10,240	10,240	4,700
16	40	80	40	40	40	160	87
17	40	40	40	40	40	40	43
18	40	40	40	40	40	40	34
19	N	10	N	N	10	P	3.1
20	40	80	40	160	160	40	69
21	160	160	160	160	160	160	151
22	N	N	N	10	10	10	4.3
23	10	40	10	10	10	10	8.3
24	160	80	640	640	640	640	290
25	40	160	160	160	160	160	80

[a]Positive reaction on the screening test at a 1-7 dilution, negative at the 1-10 dilution.

Table 10. (Continued)

Serum number	Laboratory number						Geometric mean titer
	79	14	40	59	53	19	
1	640	160	40	40	40	160	93
2	2,560	320	160	160	160	640	210
3	640	160	40	40	40	160	91
4	160	40	40	40	10	160	55
5	640	320	160	160	160	640	260
6	40	10	N	10	N	P	7.2
7	640	160	40	40	640	160	85
8	40	P	N	N	N	N	4.5
9	2,560[b]	10,240	2,560	2,560	10,240	10,240	3,700
10	10	N	N	N	N	N	3.8
11	640	160	160	160	160	640	250
12	160	40	40	40	10	40	54
13	160	20	40	40	10	40	31
14	2,560	1,280	640	640	640	2,560	1,100
15	2,560[b]	5,120	10,240	2,560	10,240	10,240	4,700
16	160	160	160	160	160	160	87
17	40	40	40	40	40	160	43
18	160	40	40	40	10	40	34
19	10	N	N	N	N	N	3.1
20	160	40	40	40	40	40	69
21	640	160	40	160	160	160	151
22	10	N	N	N	N	10	4.3
23	40	N	N	10	N	P	8.3
24	2,560	160	160	160	160	640	290
25	160	160	40	40	40	160	80

[b]These titers were not end point determinations and were not used in the calculation of the geometric mean titers.

Table 10. (Continued)

Serum number	Laboratory number						Geometric mean titer
	35	70	30	49	45	46	
1	40	40	35	160	160	640	93
2	160	160	140	160	640	2,560	210
3	40	40	35	160	640	2,560	91
4	40	40	35	160	160	2,560	55
5	160	160	140	160	640	2,560	260
6	N	P	N	10	10	2,560	7.2
7	40	40	35	160	160	640	85
8	N	P	N	10	N	2,560	4.5
9	2,560	10,240	8,960	10,240	640[b]	2,560	3,700
10	N	N	N	N	N	160	3.8
11	160	160	140	640	640	2,560	250
12	40	40	35	160	160	2,560	54
13	10	10	P	40	40	2,560	31
14	640	2,560	560	2,560	640[b]	2,560	1,100
15	640	10,240	8,960	10,240	640[b]	2,560	4,700
16	40	40	35	160	160	2,560	87
17	10	40	P	160	40	640	43
18	10	40	P	40	40	640	34
19	N	P	N	10	N	640	3.1
20	40	160	N	160	160	640	69
21	40	640	35	640	640	640	151
22	N	P	P	10	N	2,560	4.3
23	10	10	P	40	10	2,560	8.3
24	160	640	560	640	640	2,560	290
25	40	160	140	160	160	2,560	80

Table 10. (Continued)

Serum number	Laboratory number						Geometric mean titer
	71	42	28	18	32	13	
1	640[b]	160	160	160	40	160	93
2	640[b]	160	160	160	160	160	210
3	640[b]	160	160	40	40	160	91
4	640[b]	160	40	40	40	160	55
5	640[b]	640[b]	160	160	160	640	260
6	40	40	10	P	N	10	7.2
7	640[b]	160	160	160	40	160	85
8	10	N	10	N	N	10	4.5
9	640[b]	640[b]	10,240	10,240	10,240	10,240	3,700
10	10	10	N	N	N	N	3.8
11	640[b]	640[b]	640	160	160	640	250
12	160	40	160	40	40	40	54
13	160	40	40	10	10	40	31
14	640[b]	640[b]	2,560	640	640	2,560	1,100
15	640[b]	640[b]	10,240	2,560	2,560	10,240	4,700
16	160	40	40	40	40	160	87
17	160	40	640	10	40	40	43
18	160	40	40	40	40	40	34
19	N	N	N	N	N	N	3.1
20	160	40	160	40	40	160	69
21	640[b]	640[b]	160	160	160	160	151
22	10	N	10	N	N	10	4.3
23	10	N	10	P	10	10	8.3
24	640[b]	160	640	160	160	640	290
25	160	40	160	40	10	160	80

Table 10. (Continued)

Serum number	Laboratory number						Geometric mean titer
	39	23	38	11	20	33	
1	40	40	40	10	50	2,560	93
2	160	2,560	40	160	100	160	210
3	40	2,560	40	10	100	40	91
4	40	40	40	10	50	10	55
5	160	NT[c]	160	640	200	160	260
6	N	P	N	N	N	N	7.2
7	40	640	N	40	50	40	85
8	N	P	N	N	N	N	4.5
9	640	10,240	P	10,240	100	2,560	3,700
10	N	N	N	N	N	N	3.8
11	640	640	N	40	200	640	250
12	40	10,240	N	10	50	40	54
13	40	40	10	10	50	160	31
14	640	640	640	640	1,600	640	1,100
15	640	2,560	10,240	10,240	1,600	2,560	4,700
16	40	2,560	40	40	50	40	87
17	40	10	10	P	50	160	43
18	40	10	10	P	50	10	34
19	N	N	N	N	N	N	3.1
20	40	640	40	40	50	160	69
21	160	40	160	160	400	160	151
22	N	N	N	10	N	N	4.3
23	10	10	N	N	N	N	8.3
24	160	160	40	40	200	640	290
25	40	10	40	40	50	160	80

[c]No test performed on this sample.

Table 10. (Continued)

Serum number	Laboratory number						Geometric mean titer
	80	50	31	22	25	34	
1	40	160	160	40	160	40	93
2	160	160	160	160	160	160	210
3	40	160	160	40	160	40	91
4	40	40	40	40	40	40	55
5	160	640	640	160	640[b]	160	260
6	P	10	10	N	10	N	7.2
7	40	160	160	40	40	40	85
8	N	10	N	P	N	N	4.5
9	10,240	2,560	20,480	640[b]	640[b]	2,560	3,700
10	N	2,560	N	P	N	N	3.8
11	160	640	640	160	160	160	250
12	40	40	40	40	40	40	54
13	10	40	40	10	40	10	31
14	640	2,560	2,560	640[b]	640[b]	160	1,100
15	10,240	2,560	10,240	640[b]	640[b]	2,560	4,700
16	40	160	160	40	40	160	87
17	40	40	40	40	40	40	43
18	40	40	40	10	40	40	34
19	N	P	N	N	N	N	3.1
20	160	160	160	40	160	10	151
22	N	10	N	N	N	N	4.3
23	P	10	10	N	10	10	8.3
24	160	640	640	160	160	160	290
25	40	160	160	40	40	40	80

Table 11. Analysis of variance of the Stoenner plate test

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera	24	625.4214	26.0592
Laboratories	34	98.7563	2.9050
Residual	805[a]	115.8855	0.1440

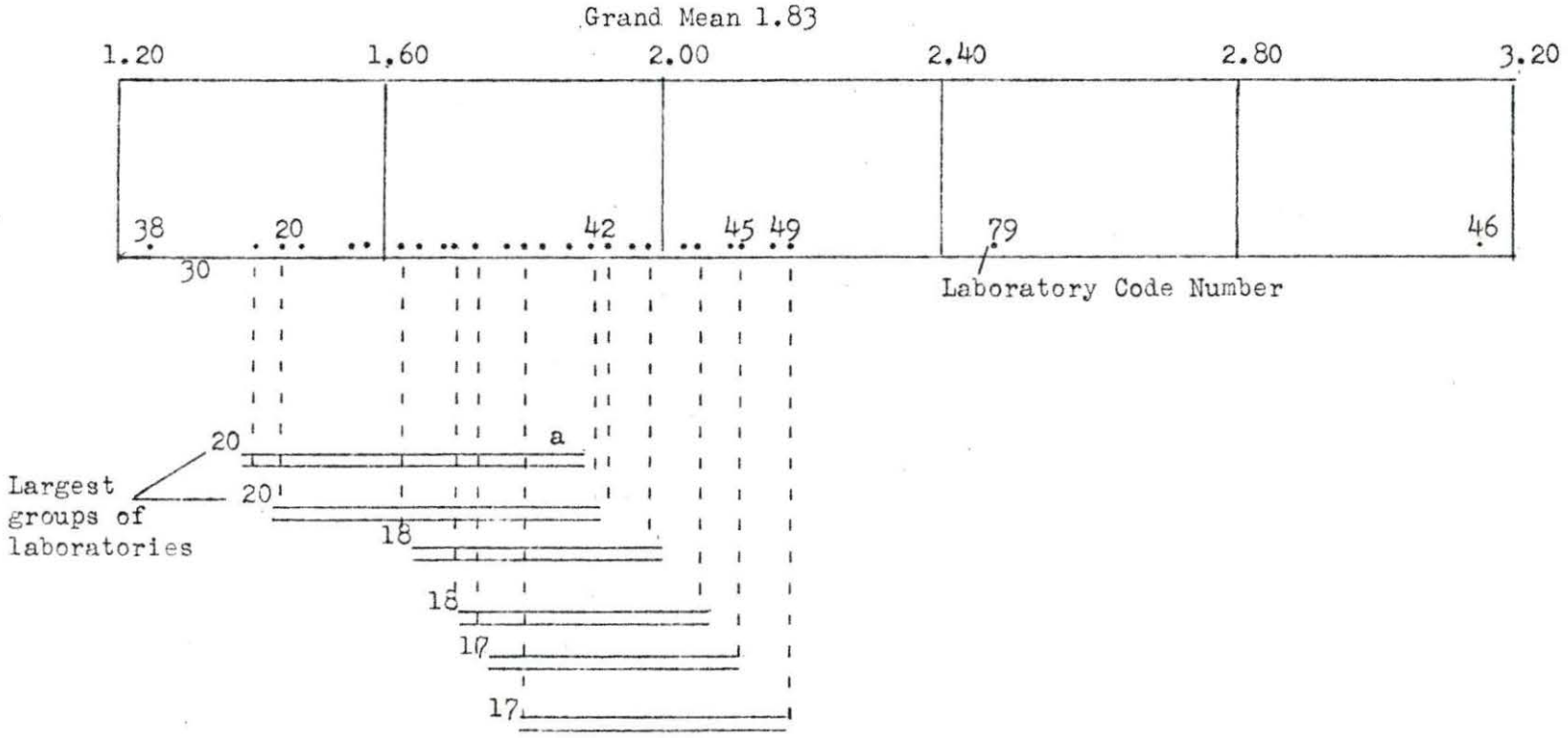
[a]Eleven degrees of freedom subtracted for missing data estimations.

Stoenner plate test was attributed to the lack of a standard dilution protocol. Three laboratories whose mean values were in the extreme range were removed from the group and the analysis of variance was repeated. An F ratio of 8.92 was then calculated which compared favorably with the Galton F ratio of 8.70. The justification for this process was the aberrant techniques reported by these laboratories. It was interesting to note, however, how three laboratories out of thirty-five were able to influence the total picture of the ability of the Stoenner plate laboratories to replicate the titers of the test sera.

Examination of the laboratory means in relation to deviation from the standard technique produced further correlation between this factor and systematic laboratory variation. Figure 3 presents the laboratory means on a linear scale with bars connecting those means not significantly different at the 5 percent level.

Except for a few laboratories, the means were fairly well grouped together with the largest group of laboratories not possessing significant

Figure 3. Laboratory means of the Stoenner plate test arranged on a linear scale.



[a]Each bar connects laboratory means not significantly different from one another at 5% level.

differences located below the grand mean. Laboratory 46, whose mean was considerably out or range, prepared its own antigen for the test. This antigen gave a 1-160 titer with the negative serum, #10. Two other laboratories, 45 and 49, whose means were in the high range, prepared their own antigen. Laboratory 79 used magnification to read the test, thereby pulling its mean into a higher value. The low mean of laboratory 38 may have been due to some clerical error, since the laboratory reported sera #6 through #12 as negative. The end titers of the rest of the sera looked quite reasonable. Laboratories 20 and 30, whose means were on the low side of the scale, used different dilution schemes than the one recommended by Stoenner. Laboratory 42 was of interest in that it reported the use of an antigen lot that was two months outdated. The mean of this laboratory, 1.85, was very close to the grand mean, 1.83.

The next question which was presented was how much of the variation among the laboratory means was due to differences in the antigen. If there were variations in the sensitivity among different lots of antigen or among antigens made by different producers, this would have increased the variation among laboratories. To check this point, the means of the laboratories employing the Stoenner Plate test were subjected to further analysis using a hierarchal classification or nested sample technique (17). This method of analysis permitted the separation of the variance between laboratory means into two separate components; one contributed by variation between producers and the other by variation among lots of antigen (antigen lot as identified by serial number). There were two producers, one represented by eleven lots and the other by

three lots. The laboratories which made their own antigen were excluded from this analysis. Table 12 summarizes this analysis of variance.

Table 12. Analysis of variance of laboratory means, Stoenner plate test

Source of variation	Degrees of freedom	Sum of squares	Mean square
Producers	1	0.0472	0.04720
Lots in Producers	12	1.0074	0.08395
Laboratories in Lots	18	0.8061	0.04478
Total	31	1.8607	

Systematic variation between antigen producers. $F = \frac{.04720}{.08395} = 0.56$, $F_{.05}=4.75$, $F_{.01}=9.33$

Systematic variation between antigen lots on the same producer. $F = \frac{0.08395}{0.04478} = 1.87$, $F_{.05}=2.34$, $F_{.01}=3.37$

The mean square for producers divided by the mean square for lots in producers yielded an F ratio which represented the systematic variation between producers. An F value of 0.56 indicated that no significant component of variance was contributed by the producers. The ratio of the mean square for lots in producers to laboratories in lots represented the component of variance contributed by differences among serial number lots of antigen. An F of 1.87 was calculated and, compared with the theoretical F of 2.34 at the 5% level, was not considered significant. From the magnitude of this F value, 1.87, it may be possible that definite differences in sensitivity occurred among the lots of antigen. However, the laboratory variation was so large that the contribution

made by the different antigen lots was small by comparison.

The systematic laboratory variation observed in the Stoenner plate test may have been due to the inadequate training of personnel. Since the antigen was commercially available, anyone could obtain it and set up a laboratory with a testing box and some pipets. However, adequate instruction under someone familiar with the technique may be necessary for accurate performance of the test.

Agglutination-lysis Test

The agglutination-lysis test has been the universal serological technique used in leptospirosis research since the early work of Schüffner and Mochtar (9). The antibody titers produced with sera from experimentally infected animals using this technique have been extremely high reaching dilutions of $1\text{-}10^8$ (18). Unfortunately, every laboratory has developed its own protocol for the test. The questionnaires submitted by the laboratories described every conceivable variation which could be introduced. Basically, the test was performed by mixing equal volumes of a living leptospiral culture and the serum dilutions, incubating for a short period of time, and examining for agglutination and lysis using a dark-field microscope. The term lysis was applied to the tightly packed masses of leptospira which appeared as bright, round balls against the dark background in the higher dilutions just before the end point titer was reached. Although any particular laboratory was capable of duplicating its own results, the replication on the test samples among laboratories was expected to be quite poor. The results of the survey are presented in Table 13.

Table 13. Agglutination-lysis test

Serum number	Laboratory number					Geometric mean titer
	81	69	9	10	63	
1	1,600	6,400	2,500	400	100,000	3,500
2	6,400	6,400	12,500	6,400	100,000	17,000
3	6,400	6,400	12,500	400	100,000	6,200
4	1,600	1,600	500	400	10,000	2,300
5	6,400	25,600	25,000	6,400	10,000	12,300
6	400	400	100	N	100	155
7	1,600	6,400	2,500	400	10,000	4,100
8	400	400	100	N	1,000	150
9	25,600	102,400	100,000	25,600	10,000,000	190,000
10	N	N	500	N	N	3
11	NT[a]	6,400	12,500	1,600	100,000	15,800
12	400	1,600	2,500	400	1,000	1,660
13	100	1,600	500	100	1,000	1,000
14	6,400	25,600	100,000	6,400	10,000	30,000
15	25,600	102,400	100,000	25,600	10,000,000	52,000
16	6,400	1,600	500	400	10,000	1,820
17	400	400	500	400	10,000	850
18	400	100	500	100	1,000	320
19	1,600	100	10	N	100	56
20	400	100	500	100	1,000	360
21	100	400	2,500	100	1,000	890
22	N	400	10	N	10	37
23	400	400	100	100	1,000	240
24	6,400	6,400	2,500	6,400	1,000	12,300
25	1,600	1,600	2,500	1,600	1,000	2,500

[a]No test reported on this serum.

Table 13. (Continued)

Serum number	Laboratory number					Geometric mean titer
	1	80	68	78	3	
1	100,000[b]	1,000	4,000	1,600	30,000	3,500
2	100,000[b]	1,000	8,000	6,400	30,000	17,000
3	100,000[b]	1,000	16,000	6,400	10,000	6,200
4	100,000[b]	1,000	4,000	400	3,000	2,300
5	100,000[b]	1,000	16,000	6,400	10,000	12,300
6	1,000	100	1,000	100	300	155
7	100,000[b]	1,000	4,000	400	10,000	4,100
8	1,000	100	500	400	300	150
9	100	1,000,000	256,000	25,600	30,000[b]	190,000
10	N	N	N	N	N	3
11	100,000[b]	10,000	16,000	1,600	30,000	15,800
12	100,000[b]	1,000	4,000	1,600	3,000	1,660
13	100,000[b]	100	2,000	400	1,000	1,000
14	100,000[b]	10,000	64,000	6,400	30,000[b]	30,000
15	10	100,000	256,000	12,800	30,000[b]	52,000
16	100,000[b]	1,000	2,000	1,600	1,000	1,820
17	1,000	1,000	500	400	1,000	850
18	1,000	100	1,000	400	300	320
19	100	10	500	400	100	56
20	1,000	100	500	400	1,000	360
21	1,000	1,000	2,000	400	1,000	890
22	100	10	100	N	100	37
23	1,000	100	500	400	100	240
24	100,000[b]	1,000	4,000	12,800	30,000	12,300
25	100,000[b]	1,000	2,000	1,600	3,000	2,500

[b]This value was not an end point titer and was not included in the calculation of the geometric mean titer.

Table 13. (Continued)

Serum number	Laboratory number					Geometric mean titer
	50	72	54	4	6	
1	3,540,000	6,400	1,000	1,000	2,560	3,500
2	39,000,000	6,400	5,000	10,000	10,240	17,000
3	39,000,000	25,600	1,000	10,000	2,560	6,200
4	322,000	6,400	1,000	1,000	640	2,300
5	322,000	25,600	5,000	1,000	2,560	12,300
6	242	1,600	N	1,000	160	155
7	322,000	409,600	5,000	10,000	640	4,100
8	242	400	N	100	40	150
9	428,000,000	409,600	50,000	100,000	163,840	190,000
10	242	N	N	N	N	3
11	39,000,000	409,600	5,000	10,000	NT	15,800
12	322,000	25,600	100	1,000	NT	1,660
13	322,000	102,400	100	1,000	NT	1,000
14	428,000,000	409,600	10,000	10,000	NT	30,000
15	428,000,000	100	50,000	10,000	NT	52,000
16	322,000	102,400	1,000	1,000	2,560	1,820
17	39,000,000	1,600	100	1,000	640	850
18	29,300	1,600	100	1,000	640	320
19	242	400	N	100	40	56
20	29,300	6,400	N	1,000	640	360
21	322,000	25,600	100	1,000	2,560	890
22	242	6,400	1,000	100	40	37
23	2,662	1,600	100	100	640	240
24	428,000,000	25,600	100,000	1,000	10,240	12,300
25	39,000,000	25,600	10,000	1,000	2,560	2,500

Table 13. (Continued)

Serum number	Laboratory number					Geometric mean titer
	77	48	74	8	45	
1	2,000	20,000	1,860	1,600	5,000	3,500
2	20,000	200,000	20,400[b]	1,600	50,000	17,000
3	20,000	20,000	1,860	1,600	50,000	6,200
4	2,000	2,000	1,860	400	5,000	2,300
5	20,000	20,000	20,400[b]	6,400[b]	500,000	12,300
6	200	200	169	100	500	155
7	2,000	20,000	1,860	1,600	50,000	4,100
8	200	2,000	169	100	500	150
9	200,000[b]	2,000,000	20,400[b]	6,400[b]	500,000	190,000
10	N	N	N	N	10	3
11	20,000	200,000	20,400	6,400[b]	50,000	15,800
12	2,000	2,000	1,860	1,600	5,000	1,660
13	2,000	2,000	1,860	400	5,000	1,000
14	200,000[b]	200,000	20,400[b]	6,400	50,000	30,000
15	200,000[b]	2,000,000	20,400[b]	6,400[b]	500,000	52,000
16	2,000	20,000	1,860	400	5,000	1,820
17	2,000	2,000	169	400	500	850
18	200	200	169	400	500	320
19	20	200	169	100	10	56
20	200	2,000	169	400	500	360
21	2,000	2,000	1,860	400	500	890
22	20	200	15	N	100	37
23	200	200	1,860	100	100	240
24	20,000	200,000	20,400[b]	1,600	500,000	12,300
25	2,000	20,000	1,860	400	5,000	2,500

Table 13. (Continued)

Serum number	Laboratory number					Geometric mean titer
	47	52	7	46	61	
1	2,000	10,000	6,400	100	800	3,500
2	200,000	10,000	51,200	1,000	3,200	17,000
3	20,000	1,000	12,800	1,000	800	6,200
4	20,000	10,000	6,400	100	1,600	2,300
5	2,000,000	10,000	12,800	1,000	1,600	12,300
6	200	100	400	10	50	155
7	20,000	1,000	6,400	1,000	800	4,100
8	200	100	800	100	100	150
9	200,000,000	100,000	N	1,000	25,600	190,000
10	N	N	N	N	N	3
11	200,000	10,000	12,800	1,000	3,200	15,800
12	20,000	100	3,200	100	800	1,660
13	2,000	1,000	51,200	100	400	1,000
14	200,000	100,000	102,400[b]	1,000	6,400	30,000
15	20,000,000	100,000	102,400[b]	10,000	25,600	52,000
16	2,000	100	12,800	100	800	1,820
17	200	100	6,400	100	400	850
18	200	100	800	100	200	320
19	200	10	100	10	25	56
20	2,000	100	1,600	10	100	360
21	2,000	100	1,600	100	200	890
22	N	N	400	100	200	37
23	200	10	400	100	200	240
24	2,000,000	1,000	51,200	1,000	3,200	12,300
25	20,000	100	12,800	1,000	800	2,500

Table 13. (Continued)

Serum number	Laboratory number				Geometric mean titer
	49	73	53	62	
1	10,000	2,000	1,250	1,600	3,500
2	10,000	2,000,000	1,250	6,400	17,000
3	10,000	2,000	50	1,600	6,200
4	100,000	20,000	50	1,600	2,300
5	1,000,000	20,000	50	6,400	12,300
6	10,000	200	N	400	155
7	10,000	2,000	250	6,400	4,100
8	100	200	10	100	150
9	1,000,000	200,000,000	250	25,600	190,000
10	N	N	N	N	3
11	10,000	2,000	1,250	1,600	15,800
12	10,000	20,000	10	1,600	1,660
13	1,000	2,000	10	400	1,000
14	1,000,000	20,000	50	6,400	30,000
15	1,000,000	2,000	250	1,600	52,000
16	1,000	2,000	50	1,600	1,820
17	10,000	200	50	400	850
18	100	2,000	N	400	320
19	100	200	N	100	56
20	1,000	200	50	400	360
21	1,000	2,000	250	400	890
22	100	20	N	100	37
23	10,000	20	50	100	240
24	10,000	2,000	1,250	6,400	12,300
25	1,000	200	250	6,400	2,500

The titers presented in the table are all based on a 50 percent end point. Two laboratories reported different titers than would have been achieved by following the dilution protocols described in their questionnaires. Another laboratory reported 25 percent end point titers. The readings of these three laboratories were corrected to correspond with the other laboratories. The variation in titer values on the same serum are almost incredible. Serum number 9 shows a hundred thousand fold difference between two readings reported by different laboratories. The analysis of variance was performed and the results are presented in Table 14.

Table 14. Analysis of variance of the agglutination-lysis test

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera	24	877.9600	36.5817
Laboratories	27	387.4342	14.3494
Residual	635[a]	320.6723	0.5050

[a]Eleven degrees of freedom subtracted for missing data estimations.

The F ratio for laboratories variation was calculated to be 28.41 as against a theoretical F of 1.81 at the one percent level. This value indicated great systematic variation in how the technique was reproduced among laboratories. The standard deviation calculated from the residual was a dilution factor of 1 : 5.14 indicating a higher degree of experimental error than the twofold dilution factor which was observed in the other techniques. Replication runs on the test sera

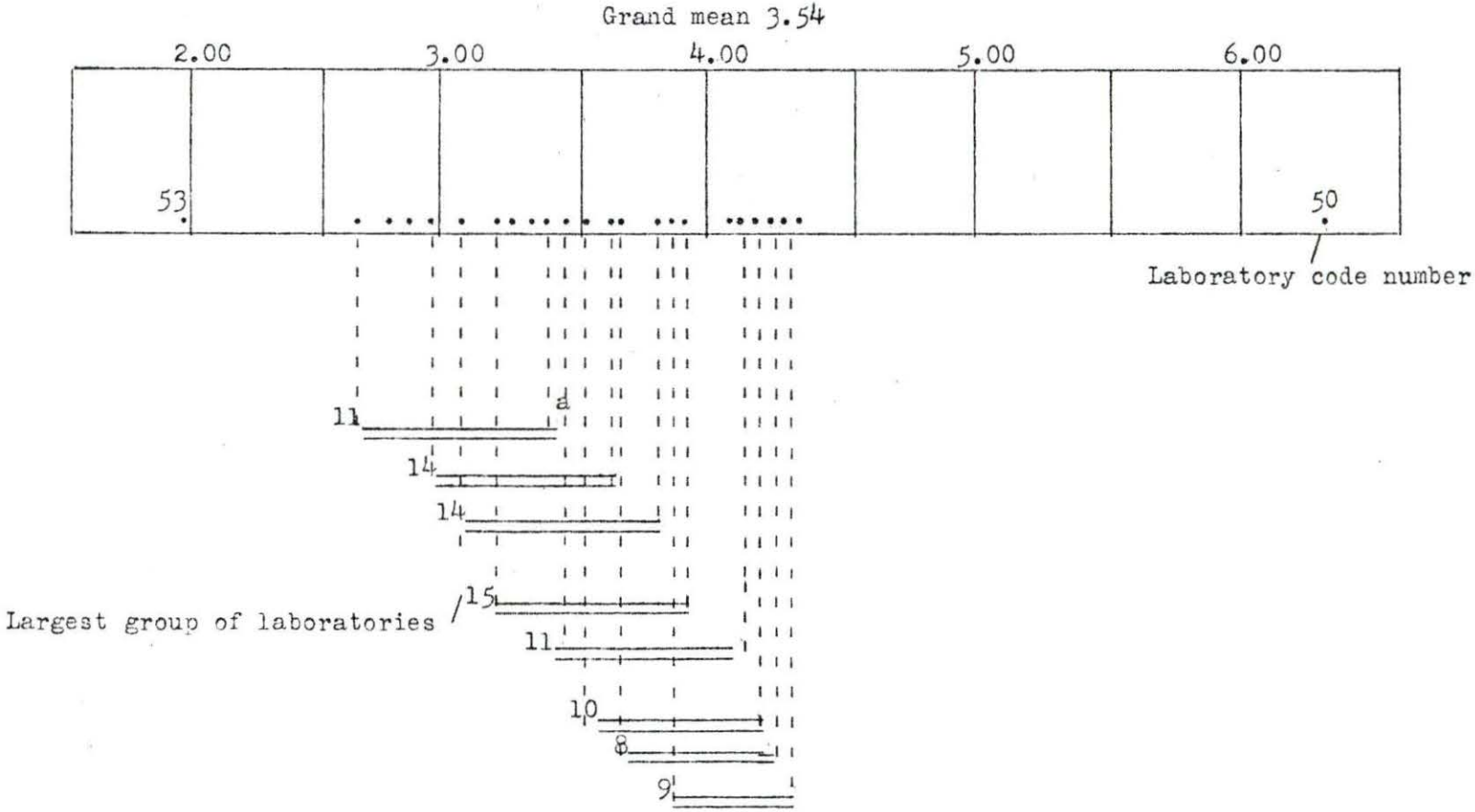
performed by the author resulted in the much lower value of a 1 : 1.88 dilution factor.

It was obvious that the agglutination-lysis test needed some efforts towards standardization. However, which factors were agents of variation in the test or which laboratories were responsible was the question. The linear arrangement of laboratory means with the application of the Q test as presented in Figure 4 demonstrated the extreme values of laboratories 53 and 50.

Unlike the other serological tests the largest group of laboratories, fifteen, were located symmetrically about the grand mean. Laboratory 53, at the lower end of the scale, employed a strain of L. pomona for antigen which had been isolated at that laboratory. It was not stated in the questionnaire whether this strain, named Schlitz, had been checked out by one of the leptospirosis reference laboratories. Laboratory 50 at the extreme upper end of the scale used a greater than tenfold dilution scheme which involved the use of a 0.2 ml. pipet transferring 0.01 ml. aliquots. The rinsing of serum proteins from this small pipet and the carrying of significant amounts of inoculum on the external surface of the pipet may have contributed to the high mean of this laboratory.

The questionnaires submitted by the laboratories with the test results were used to gain some indication as to which variables in the agglutination-lysis test caused significant differences between laboratories. The standardization of the live antigen used in the test was accomplished by dark-field examination in twenty-six out of twenty-eight laboratories. Luxuriant cultures were diluted to obtain an antigen of suitable density as observed by either high or low power.

Figure 4. Arrangement of laboratory means on a linear scale, agglutination-lysis test.



[a]Each bar connects laboratory means not significantly different from one another at 5% level.

One laboratory reported standardization of the antigen by nephelometry and another laboratory used a Petroff-Hausser counting chamber to count the leptospirae in their antigen. About half of the laboratories reported the use of centrifugation to "clear" the antigen. Microscopic magnification employed to read the test varied from 100X to 250X. All types of microscopes were used including the improvised dark field with a bright field condenser. Methods of cleaning glassware varied considerably. However, all laboratories agreed on the need for adequate rinsing with tap water followed by rinsing with ion exchange or a similar grade of distilled water. A preference was shown for alkaline detergent washing compounds. Four factors were distributed among the laboratories so that comparisons could be made among laboratory means. These factors were the dilution scheme used to prepare the serum dilutions, the antigen strain of L. pomona employed, the temperature used to incubate the serum dilution-antigen mixtures, and the culture medium employed to grow the antigen.

Ideally a study of these four factors as sources of variation should have been carried out in one large analysis comprising all four factors, but with the incomplete data presented such an analysis would have been extremely involved. Instead two factor tables were prepared such as antigen strain versus dilution scheme comparing these factors. A preliminary check of the variance between the blocks of these tables revealed only one which approached significance (Table 15).

The following F ratio was calculated:

Blocks vs Residual: $F = 2.44$, $(F_{.05} = 2.67)$

Table 15. Preliminary analysis of variance of the two factor table of laboratory means classified by antigen strains and culture media

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between blocks	10	8.1146	0.81146
Residual	13	4.3179	0.33215

Since the F ratio was close to the 5 percent level a further analysis was performed to calculate the components of variance due to antigen strain, culture media and interaction. This involved the solving of eight simultaneous equations in seven unknowns to correct for the uneven numbers of laboratory means in each block (19). There were three culture medium classes: Stuart's medium, Schuffner's medium, and miscellaneous media. The antigen strain employed by each laboratory divided the means into four classes: Pomona, Johnson, S-91, and miscellaneous strains. In table 16 the analysis is summarized.

The following F ratios were calculated from the table:

Culture media alone $F = 1.50$, $F_{.05} = 3.80$
Antigen strains alone $F = 2.02$, $F_{.05} = 3.41$
Interaction $F = 3.35$, $F_{.05} = 3.02$

According to this evaluation neither antigen strains alone nor culture media along were significant sources of variation. The F for interaction was above the 5 percent level and it would be reasonable to expect that growth of a strain in different culture media might have some effect on its antigenicity. However, the table contained two miscellaneous classes and this casts a degree of doubt on any conclusion drawn from it.

Table 16. Analysis of variance of laboratory means classified by antigen strains and culture media

Source of variation	Degrees of freedom	Sum of squares	Mean square
Antigen strains alone	3	2.0188	0.67293
Between culture media (estimation of constants)	2	0.5277	
Antigen strains and culture media (estimation of constants)	5	2.5465	
Interaction	5	5.5681	1.11362
Between antigen strains (estimation of constants)	3	1.5490	
Culture media alone	2	0.9975	0.49875
Residual[a]	13	4.3179	0.33215

[a]Residual taken from Table 15.

A direct comparison of means using the "t" test was applied to the classes of each of the four factors. This method ignored the effect of the other factors but was the only effective way to handle the data. Class means were calculated for each subdivision of laboratories within a factor and compared. Table 17 presents the separation by dilution scheme.

The array suggested a direct comparison between tenfold and the other two dilution schemes. In Table 18 this comparison is shown. Three sample standard error ($S_{\bar{x}}$) values were obtained from the two-way factorial tables previously described and a "t" value was calculated with each one. In this comparison all probabilities determined were

Table 17. Comparison by dilution scheme

Dilution scheme	Number of laboratories	Mean
Twofold	3	3.333
Fourfold	8	3.264
Tenfold	13	3.926

Table 18. Limited comparison by dilution scheme

Dilution scheme	Number of laboratories	Mean
Tenfold	13	3.926
Twofold and fourfold	11	3.283
	Difference	0.643

less than 5 percent, indicating a significant contribution to laboratory variation by the use of different dilution schemes.

The t values calculated for the above difference using the three sample standard errors indicated probabilities of 0.010, 0.013 and 0.025.

Means were calculated for laboratories using the same antigen strain and compared. The mean for the Johnson strain was 3.958, whereas the means of the Pomona strain, S-91 strain, and miscellaneous strains were 3.396, 3.472, and 3.443, respectively. This suggested a direct comparison for significance of the difference between the Johnson strain and the other strains as a group, as shown in Table 19.

The "t" values calculated for the difference gave rise to

probabilities of 0.005, 0.022, and 0.070. Although antigen strains were found to be significant sources of variation by the author in controlled experiments the higher sensitivity of the Johnson strain was not substantiated. The possibility must be conceded that the above result was due to some unknown associated agent.

Table 19. Comparison by antigen strain

Antigen strain	Number of laboratories	Mean
Johnson	9	3.958
Pomona, S-91, and others	15	3.435
	Difference	0.523

Three different temperature ranges were employed by the laboratories in the survey to incubate the serum dilution-antigen mixtures: room, 28°-32°C, and 37°C. The mean calculated for the 37° group was 4.013 in comparison with the means of the room temperature and 28°-32° groups which were 3.569 and 3.401, respectively. A direct comparison was made between 37° and room temperature combined with the 28°-32° group which produced a difference of 0.509. The t calculated from this value was significant at probability levels of 0.041, 0.066, and 0.078. The conclusion that a higher incubation temperature would increase the end point titers of a group of sera was a reasonable one and was indicated by the survey data. Controlled experiments reported later in this thesis found increased titers related to higher incubation temperatures but not at a significant level of probability.

A comparison was made of the means of laboratories using different culture media and no significant differences were found. Eleven laboratories employed Stuart's medium, eight laboratories used Schuffner's, two laboratories used Chang's, and the remaining three laboratories employed a medium of their own invention.

DISCUSSION

From the survey data it may be concluded that great variation in the application of serological techniques for the diagnosis of leptospirosis existed among laboratories. In regard to the tests such as the Stoenner and Galton, which have been carefully described and for which a source of commercial antigen is available, emphasis must be placed on strict adherence to protocol. It might be of further assistance for some qualified person to visit each laboratory and encourage steps in this direction. The agglutination-lysis test, if it is to be used as a diagnostic tool, must be standardized by some agency which will exert effective influence on the laboratories concerned. It is unfortunate that the cumbersome nature and infection hazard of this technique will prevent its universal adoption.

Correlation studies performed on the serum means from the survey data revealed excellent correlation between the killed antigen tests and the agglutination-lysis test. The sample correlation coefficients for the tests compared with the agglutination-lysis test were as follows:

Stoenner plate test,	$r = 0.923$
Stoenner capillary tube test,	$r = 0.902$
Galton plate test,	$r = 0.900$
Cox hemolytic test,	$r = 0.789$

It would appear that all of these tests were measuring the same antibody response. Using the grand means from the survey for each test it was found that an agglutination-lysis titer was roughly 26 times greater than a Stoenner plate titer, 18 times a Stoenner capillary tube titer, 60 times a Galton plate titer, and 51 times a Cox hemolytic

titer. Concerning the two plate techniques the Stoenner titer was 2.34 times the Galton plate titer. Although these relationships might be used to transform the titers of one test into those of another, it would be a better solution to adopt one specific technique as an official test and ruthlessly eliminate the others.

One of the problems of leptospirosis diagnosis today is the interpretation of serological titers. The desire to create an analogy with the "reactor" titer of the brucellosis test is both natural and tempting. A "reactor" titer for leptospirosis diagnosis would furnish a necessary constant for the establishment of regulations. Unfortunately, the relation of a particular antibody titer to the infective stage of the disease has not been decided to the satisfaction of everyone. From the data accumulated in this survey, we can approach this problem from a different angle. The standard deviation of a serological technique represented its inherent experimental error as it was applied by all of the laboratories. Now, if a titer were selected which was generally considered a nonspecific level of antibody, the standard deviation may be used to calculate a minimum titer value which would be based on the experimental error of the technique. If the minimum titer were selected so that it was exactly two standard deviations above the nonspecific titer level, then a 95% confidence interval would have been created. When this minimum titer is observed as the reacting titer there would still be a one in forty chance of incorrectly designating an animal as infected when the antibody content of the serum is actually at the nonspecific level. The minimum titers for four of the tests have been calculated and are shown in Table 20.

Table 20. Minimum titers at a 95 percent confidence level based on standard deviation of the test

Test	Standard deviation[a]	Nonspecific titer	Minimum titer[b]
Agglutination-lysis	5.14	100 (25)	1028 (257)
Stoenner plate	2.40	10	48
Stoenner capillary tube	2.81	10	56
Galton plate	2.53	11.9	60

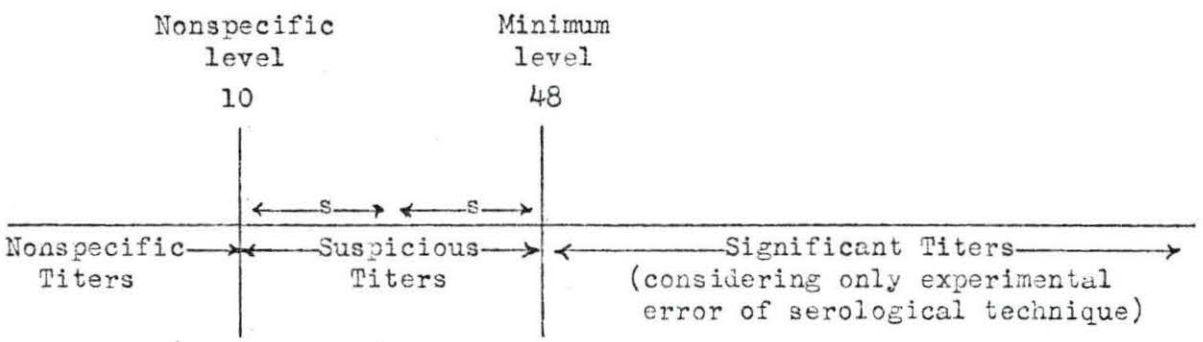
[a]Expressed as a dilution factor.

[b]Twice standard deviation above nonspecific titer.

Of course, these minimum titers have no relation to the biological picture, but are based simply on the experimental error of the serological techniques. The nonspecific titer levels were selected arbitrarily and are open to criticism. Using the standard deviation for the agglutination-lysis test of a 5.14 dilution factor, selection of a nonspecific titer level of a 1:100 dilution produced a minimum titer at the 1:1028 dilution. An alternative minimum titer of 1:257 was computed for the lower nonspecific level of 1:25. The minimum titers for the two Stoenner tests were calculated to be 1-48 and 1-56 which were quite close to the standard 1-40 dilution used in these techniques. The diagram in Figure 5 shows the relation of the two titer levels in the Stoenner plate test.

Titers in the range between the nonspecific titer and the minimum titer may be classified as suspicious.

Figure 5. Stoenner plate test range.



s = standard deviation

The proposition of the minimum titer as outlined above may be somewhat removed from the natural aspects of the disease and can only serve as an expression of the limitations of our serological techniques for the diagnosis of leptospirosis. However, it seems clearly indicated that the reporting of serological titers lower than these minimal titers as positive indications of the disease serves to create false impressions. The reacting titer may be placed anywhere above this minimum level, but certainly cannot be placed below it without implying an accuracy which does not exist in the serological tests employed.

SUMMARY

A survey of the serological techniques used for the diagnosis of leptospirosis was made by sending sets of twenty-five sera to seventy-three laboratories. A questionnaire was completed by each laboratory giving the details of the technique or techniques used. An analysis of variance performed on the data revealed a high degree of systematic variation between laboratories on the five techniques most widely used:

Agglutination-lysis
Stoenner plate
Stoenner capillary tube
Galton plate
Microscopic agglutination with formalin-treated antigen

The lowest degree of systematic laboratory variation was shown by the laboratories performing the Galton test.

The standard deviation calculated for the agglutination-lysis test was about a fivefold dilution factor. The standard deviations of the other tests were roughly the same, being slightly more than a twofold dilution factor.

The distribution of the laboratory means of each test were studied and the groups of laboratories whose means were not significantly different from one another were identified.

Analysis of variance on the laboratory means of the Stoenner plate test was performed and the components of variation contributed by the different producers and different serial lots of antigen were calculated. Neither of these sources introduced significant variation.

Statistical study of the results of the survey of the agglutination-lysis test revealed that the use of different dilution schemes, antigen

strains, and incubation temperatures produced significant components of the variation between laboratories.

A minimum titer was computed for each of the serological tests above which a high degree of confidence could be expressed in a positive interpretation of the test. This minimum titer level was located two standard deviations above the nonspecific titer level.

PART II. SOURCES OF VARIATION IN THE AGGLUTINATION-LYSIS AND STOENNER TESTS

METHODS AND MATERIALS

Antigen for the Stoenner test was obtained from Fort Dodge Laboratories, Inc., Serial Number 306124, and the test was performed according to the protocol described by Stoenner (7). To attain the serum dilution titers described in the protocol dilutions were prepared using a 0.2 ml. brucellosis pipet transferring aliquots of 0.2 ml. of the serum dilution. The first dilution tube contained 0.8 ml. of saline and the remaining tubes contained 0.6 ml. After each dilution was thoroughly mixed with the pipet, 0.03 ml. was placed on a glass plate before carrying the 0.2 ml. to the next dilution tube. The same pipet was used for all of the dilutions of each serum. The serum dilution-antigen mixtures were incubated at room temperature in a covered testing box.

The Stoenner capillary tube test was performed as previously described (8).

In all of the experiments on the agglutination-lysis test except the one comparing antigen strains L. pomona Pomona, the type strain, was employed. This strain as well as the other strains utilized were obtained from the WHO/FAO Leptospirosis Reference Laboratory, Division of Veterinary Medicine, Walter Reed Army Institute of Research, Washington, D. C. In the experiment comparing antigen strains two additional strains of L. pomona were employed: S-91 and Johnson. Leptospiral strains used as antigens were maintained in Stuart's medium (20) as prepared by Difco Laboratories, Inc. and transferred every 4 to 6 days. Approximately 9 ml. of medium were placed in sterile

screw-cap vials, 20 x 125 mm. in size. An inoculum of 1 ml. of an actively growing culture was added to each tube and the cultures were incubated for 4 to 6 days at 30°C. The antigen cultures were examined macroscopically for growth and, if suitable, were centrifuged at 500 G on a horizontal head for ten minutes. The antigen was removed from the culture tube and examined for density with the dark-field microscope at a magnification of 150X. The antigen was diluted with buffered saline until it reached the optimum level of density as determined by microscopic observation. The optimum level was defined as a density which permitted the easy determination of fifty percent end point. The L. pomona Pomona strain as cultivated usually required the addition of 4 to 6 ml. of buffered saline to 10 ml. of culture for adjustment to the correct density. The formula for the buffered saline was as follows:

Na_2HPO_4	2.0 grams
KH_2PO_4	0.4 grams
NaCl	4.0 grams
H_2O (ion exchange)	1.0 liter

On one occasion the antigen prepared in this manner was counted with a Petroff-Hausser counting chamber. The number of leptospira per milliliter was found to be 129,000,000 with a standard deviation of $\pm$37,300,000.

Serial dilutions were made in buffered saline and 0.1 ml. of each dilution was placed in a small glass tube, 10 x 75 mm., with 0.1 ml. of antigen. Titers reported in each experiment were final dilution titers achieved after the addition of antigen to the serum dilution. The tubes were shaken and incubated for two hours at 37°C., unless otherwise stated. The tests were read by placing a small

oblong drop on a microscopic slide from each tube and examining by dark-field microscopy at 150X magnification. The degree of agglutination was read as negative, 1, 2, 3, and 4. The reading corresponded to the following degrees of agglutination as judged by the number of free leptospirae rather than the agglutinated masses present:

trace percent or less	N
25 percent	1
50 percent	2
75 percent	3
100 percent	4

The 50 percent end point titer was the highest titer showing at least a 2 agglutination reading.

Since the investigations were concerned with the effects of the variable factors of the agglutination-lysis test further innovations were introduced as required in each experiment. The fundamentals of the agglutination-lysis technique employed were patterned after the method used at the WHO/FAO Leptospirosis Reference Laboratory, Walter Reed Army Institute of Research (21).

RESULTS

Replication of the Stoenner Plate Test

The Stoenner plate test was performed on the twenty-five test sera used in the survey to determine the reproducibility of this technique as applied within one laboratory. A series of five complete determinations was made from start to finish. The titer values were converted to logarithms as previously described and subjected to analysis of variance (Table 21).

Table 21. Analysis of variance on the Stoenner plate test, five complete runs on twenty-five sera

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera	24	116.3421	4.8476
Runs	4	0.1776	0.0444
Residual	96	1.3238	0.0138

The F calculated for sera variance was 351 ($F_{.01} = 1.98$) reflecting the difference in the titers of the sera which was part of the experimental design. The runs F was 3.22 ($F_{.05} = 2.46$, $F_{.01} = 3.51$) which indicated a probability of about 3 percent that the variation between the runs was due to chance alone. The standard deviation calculated from the residual and expressed as a dilution factor was 1 : 1.31. The F ratio for runs was an indication of the better replication found within one laboratory when compared with the F ratio of 20.18 found for variation among laboratories in the survey data. It may also be

considered a normal indicator of the reproducibility of this biological technique. On the other hand, the residual was quite small which would seem to indicate a small experimental error of the technique. Certainly, the reading of end points as "visible agglutination" was an all or none method. Any agent acting to increase or decrease the titer would make itself felt in the end point titer of every serum. This would greatly increase the effect of the agent over that found in a test such as the agglutination-lysis test where the end point was shaded from a reading of 1 to 4.

Replication of the Agglutination-lysis Test

The agglutination-lysis test was first evaluated for reproducibility in an experiment where the tenfold dilution scheme was employed. This was the dilution scheme used by the largest number of laboratories in the survey. The negative serum, number 10, was excluded from the test sera since it has previously been determined negative in a dilution of 1 : 2. Four separate and complete runs were made on the remaining twenty-four sera on four different days. Each run represented a new lot of antigen and a new set of dilutions. The tests were read as previously described at a magnification of 100X. The analysis of variance of the data is presented in Table 22.

The runs variance was less than the residual variance in this experiment. The standard deviation was a dilution factor of 1: 2.30 which was much less than the 1 : 5.14 dilution factor determined from the survey data. It was considered that the tenfold dilution scheme employed did not adequately test for differences in the antigen lots

Table 22. Analysis of variance on the agglutination-lysis test, four complete runs on twenty-four sera

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera	23	107.9583	4.69384
Runs	3	0.2083	0.06943
Residual	67[a]	8.7917	0.13122

[a]Two degrees of freedom deducted for missing data corrections.

so another experiment was performed using a fourfold dilution scheme.

Two factors were compared simultaneously on sixteen of the sera from the survey. Four lots of antigen were prepared as described on four successive days. The cultures used to prepare the antigen were 4 to 6 days old. Each day two sets of dilutions were prepared so that two complete tests would be performed on the same lot of antigen. The two sets were identified as "1" and "2" since there was a time lapse between the preparation of the tests from each set during which the antigen remained exposed to air contamination at room temperature. The layout of the experiment is presented in Table 23.

The titers of the 16 sera were transformed into logarithms and the analysis of variance was performed as described by Kendall (19). Table 24 shows this analysis of variance.

The interaction variances were not significant compared to the residual variance, therefore, they were combined with the residual variance as in Table 25.

The F ratio calculated for antigen lots was 0.70 ($F_{.05} = 2.70$)

Table 23. Layout of experiment comparing variation between sera, antigen lots, and dilution sets presenting the results of three sera

Sera[a]	Antigen lot							
	I		II		III		IV	
	Dilution set		Dilution set		Dilution set		Dilution set	
	1	2	1	2	1	2	1	2
4[b]	1600	400	1600	1600	1600	1600	1600	1600
6	100	400	100	100	100	400	400	400
11	6400	6400	6400	6400	6400	6400	6400	6400

[a]A total of 16 sera were used.

[b]The sera were coded to conceal their identity.

Table 24. Analysis of variance on experiment combining serum, antigen lot, and dilution scheme effects

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera (S)	15	101.21257	6.74750
Antigen lots (A)	3	0.15863	0.05288
Dilution sets (D)	1	0.10069	0.10069
Interaction (SA)	45	2.09591	0.04658
Interaction (SD)	15	1.79082	0.11939
Interaction (AD)	3	0.10106	0.03369
Residual (SAD)	45	4.13351	0.09186

Table 25. Simplified analysis of variance on experiment combining serum, antigen lot, and dilution scheme effects

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera	15	101.21257	6.74750
Antigen lots	3	0.15863	0.05288
Dilution sets	1	0.10069	0.10069
Residual	108	8.12131	0.07520

and the F ratio for dilution sets was 1.33 ($F_{.05} = 3.94$). Neither factor was a source of significant variation in this experiment or, as an extrapolation, in the test as performed within one laboratory. The standard deviation in this experiment was a 1 : 1.88 dilution factor. It would appear that the agglutination-lysis test is an excellent serological technique for use in a research laboratory where one person standardizes all of the antigen and reads all of the tests.

Effect of Antigen Density

Having established the reproducibility of the agglutination-lysis technique it was deemed advisable to study the influence on the serum titer of some of the factors which produced significant variation in the survey data. However, the work of Borg-Petersen and Fagraeus (2) on the effect of antigen density suggested an effort to confirm their findings.

A master set of fourfold dilutions was prepared using fourteen sera. The final dilution titers were 25, 100, 400, 1600, 6400, 25,600, 102,400, and 409,600. Four tests were prepared from the master dilution set using different densities of the antigen culture. A luxuriant, four day old culture was diluted 1: 2, 1 : 4, and 1 : 8. The final antigen dilution in the four sets of tests prepared was 1 : 2, 1 : 4, 1 : 8, and 1 : 16. The tests were incubated and read in the usual manner except that the sera were coded.

Inasmuch as the usual antigen density used corresponded to a final dilution of about 1 : 3, the 1 : 2 and 1 : 4 antigen dilutions presented no problem. However, it must be admitted that the tests

prepared with the 1 : 8 and 1 : 16 antigen dilutions were difficult to read. The results were subjected to statistical analysis as summarized in Table 26.

Table 26. Analysis of variance on the effect of antigen density

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera	13	53.67714	4.12910
Antigen dilution	3	1.37274	0.45758
Residual	39	3.16648	0.08119

Antigen dilution was found to contribute a highly significant component of variation as indicated by an F of 5.64 ($F_{.01} = 4.34$). The means corresponding to the 1 : 2, 1 : 4, 1 : 8, and 1 : 16 antigen dilutions were 3.25, 3.16, 3.46, and 3.55, respectively; indicating a trend toward higher serum titers with higher antigen dilution. A sample regression coefficient was calculated of 0.40 log serum titer units per log unit change in antigen dilution. In other words, a tenfold increase in antigen dilution produced a 2.5 fold increase in serum titer. An F value of 12.42 was calculated for "linear trend relation" ($F_{.01} = 7.33$). This compared favorably with an F value of 2.25 ($F_{.05} = 3.24$) determined for the "deviations from linear trend" in the data. It was interesting to note that Borg-Petersen and Fagraeus (2) plotted the $\log_2$ of serum titer against $\log_2$ of antigen density and found the slope of the line connecting the points to be 0.41.

Effect of Serum Dilution Scheme

From the survey data it was concluded that the means of the laboratories using the tenfold dilution scheme were significantly higher than the means of the other laboratories. An experiment was set up to check this finding and at the same time check on the variation occurring beyond the dilution scheme. A large batch of antigen was prepared and seventeen sera were employed. Three sets of dilutions were prepared: a tenfold scheme starting with a 1 : 10 dilution, a fourfold scheme starting with a 1 : 25 dilution, and a twofold scheme starting with a 1 : 25 dilution. All sera were carried to end point titers in all dilution schemes. Two sets of tests were prepared for each dilution scheme so that the variation introduced by the mixing of equal amounts of serum dilution and antigen and the variation contributed by the reading of two sets by the same observer could be determined. The two sets were identified as "1" and "2" since there was a time lapse between the preparation of the test sets during which the antigen remained exposed to air contamination at room temperature. The experiment was set up as in Table 27.

Table 27. Layout of experiment comparing variation between sera, dilution schemes, and sets of tests presenting the results of four sera

Sera[a]	Dilution scheme					
	Twofold test set		Fourfold test set		Tenfold test set	
	1	2	1	2	1	2
4	1,600	1,600	1,600	1,600	1,000	1,000
6	200	200	400	100	100	100
7	1,600	1,600	6,400	6,400	1,000	10,000
9	102,400	102,400	409,600	409,600	100,000	100,000

[a]A total of 17 sera were used.

The serum titers were transformed into logarithms and adjusted to a logarithmic mid-point between the 50 percent end point titer and the next dilution titer. From a logical point of view stating a 50 percent end titer was actually saying that the 50 percent end point occurred between the titer value specified and the next dilution titer employed. The results were analyzed and presented in Table 28.

Table 28. Analysis of variance on experiment combining the effects of serum, dilution scheme, and test set

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera (S)	16	71.3656	4.4604
Dilution schemes (D)	2	7.9581	3.9790
Test sets (T)	1	0.0492	0.0492
Interaction (SD)	32	3.4321	0.1073
Interaction (ST)	16	1.9722	0.1233
Interaction (DT)	2	0.4669	0.2334
Residual (SDT)	32	3.7029	0.1157

Since the interactions were not significant they were combined with the residual in Table 29 (Interaction DT, $F = 2.01$, $F_{.05} = 3.30$).

Table 29. Simplified analysis of variance on experiment combining the effects of serum, dilution scheme, and test set

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera	16	71.3656	4.4604
Dilution schemes	2	7.9581	3.9790
Test sets	1	0.0492	0.0492
Residual	82	9.5741	0.1168

An extremely high F value of 34.07 ($F_{.01} = 4.88$) was found for dilution schemes which indicated the importance of this factor in contributing to variation in the agglutination-lysis test. However, the F ratio for test sets was not significant. This displayed the ability of an observer to replicate readings of the test. It also showed that the variation introduced in preparing the serum dilution-antigen mixtures was of no importance.

Since the comparison of dilution scheme means obtained from the survey data suggested no significant difference between the twofold and fourfold means the data from this experiment were subjected to the Q test (17). The results are shown in Table 30.

Table 30. Q test for comparison of dilution scheme means

Dilution scheme	Mean $\bar{x}$	$\bar{x} - 3.300$	$\bar{x} - 3.751$
Tenfold	3.970	0.670	0.219
		(0.199)	(0.165)
Fourfold	3.751	0.451	
		(0.165)	
Twofold	3.300		

All three dilution scheme means were significantly different from one another at the 5 percent level of probability. It would appear that comparison of test results should not be considered when different dilution schemes have been employed. Granted a specific content of antibody in a serum capable of dilution to a particular end point titer, the use of a different pipet for each transfer would consistently achieve this titer. However, economical serology requires the use of

the same pipet in preparing all of the dilutions for a serum. In reaching a given dilution level the pipet will receive more rinsings in preparing twofold dilutions than preparing tenfold dilutions. As an example, five bovine sera containing high antibody levels were diluted according to the fourfold scheme using first, the same pipet, and second, using a different pipet for each transfer. The end point titers are presented in Table 31.

Table 31. Comparison of same pipet versus different pipet using the fourfold dilution scheme

Sera[a]	Fourfold dilution scheme	
	Same pipet	Different pipet
8	6,400	1,600
9	6,400	400
22	6,400	1,600
23	25,600	1,600
27	25,600	1,600

[a]These sera were not from the survey group.

It was observed that frequently a sixteenfold difference in end point titer resulted when a different pipet was used for each mixing and transfer. Naturally, a serum such as survey serum number 9 which came from an animal suffering from chronic interstitial nephritis would rinse rather slowly from the inner surface of the pipet. In fact, some laboratories reported trace agglutination to the last dilution employed in their protocol when reporting the titer of serum number 9.

Effect of Test Incubation Temperature

The survey data suggested that the incubation temperatures of the test was a factor producing significant variation in serum titer. Although the antigen-antibody combination would be accelerated by higher temperatures it was not so reasonable that the end point titer of the serum would be affected. Particularly would this be true if the time of incubation were of sufficient duration. The laboratories in the survey agreed almost unanimously on an incubation time of two to four hours. An experiment was designed to check this survey finding. A master set of fourfold dilutions was prepared using sixteen sera. Three sets of tests were set up on the same batch of antigen. The first set was incubated at 23°C. representing an average room temperature. The second set was incubated at 30°C. and third set at 37°C. All three sets were incubated for a period of exactly two hours and promptly read. The means for each temperature set increased from the lowest temperature to the highest. The means were 2.978 for 23°C., 3.091 for 30°C., and 3.166 for 37°C. Although the differences between the means were small an analysis of variance was performed to check for significance (Table 32).

Table 32. Analysis of variance of the effects of test incubation temperature

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera	15	38.2099	2.54732
Temperatures	2	0.2870	0.14035
Residual	30	2.1291	0.07097

The F ratio for temperatures was 1.98 as compared to theoretical values at the 5 and 1 percent levels of 3.32 and 5.39. It was concluded that the test incubation temperature had not been a significant source of variation in this experiment. However, a trend towards higher serum titers was related to higher test incubation temperatures. The effect of temperature observed in the survey data may have been due to confounding with other factors.

Effect of Antigen Strain and Test Reader

The many strains of L. pomona were so classified if after cross-absorption with L. pomona Pomona, the type strain, less than 10 percent of the homologous titer remained in each antisera (22). This classification permitted a definite degree of antigenic variation in all strains of the serotype L. pomona. Accordingly it was not surprising to find significant variation due to antigen strain differences in the survey. Three strains of L. pomona were compared on one master set of fourfold dilutions of fourteen sera. The antigen cultures were harvested and standardized to the same density as previously described. The three strains most commonly employed in the survey Pomona, Johnson, and S-91 were used.

Since two dark-field microscopes were available the experiment was designed so that the variation produced by different test readers might be determined. Two sets of tests were prepared with each antigen strain and read simultaneously after incubation at 37°C. for two hours. The microscopes used were a Bausch and Lomb equipped with an oil dark field and 100X magnification employed by the author and a Leitz Ortholux

with a dry dark field and 150X magnification employed by Helen Sailsbury, Technician, Animal Disease Eradication Diagnostic Laboratory. Mrs. Sailsbury had been trained by the author and was routinely employed reading agglutination-lysis tests for diagnostic purposes. Many individual comparisons in the past had not revealed any obvious differences in reading technique. The layout of the experiment is presented in Table 33.

Table 33. Layout of experiment comparing sera, antigen strains, and readers presenting the results of four sera

Sera[b]	Antigen strain[a]					
	Pomona reader		Johnson reader		S-91 reader	
	EC	HS	EC	HS	EC	HS
16	400	400	400	100	400	400
13	400	400	400	400	400	400
4	400	400	400	400	400	400
12	400	400	400	400	1600	400

[a]The antigen strains and sera were coded so that no mental hazard existed for the readers.

[b]A total of 14 sera was used.

An analysis of variance was performed comparing the three factors: sera, antigen strains, and readers (Table 34).

None of the interactions approached the significant level. For interaction between sera and antigen strains an F was calculated of 1.57 ($F_{.05} = 1.94$). The analysis of variance for main factors was condensed by combining the interaction terms with the residual. This is shown in Table 35.

Table 34. Analysis of variance on the experiment combining serum, antigen strain, and reader effects

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera (S)	13	44,98985	3.46076
Antigen strains (A)	2	0.78487	0.39243
Readers (R)	1	0.97072	0.97072
Interaction (SA)	26	1.86320	0.07166
Interaction (SR)	13	0.53936	0.04149
Interaction (AR)	2	0.02572	0.01286
Residual (SAR)	26	1.18235	0.04548

Table 35. Simplified analysis of variance on the experiment combining serum, antigen strain, and reader effects

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera	13	44.98985	3.46076
Antigen strains	2	0.78487	0.39243
Readers	1	0.97072	0.97072
Residual	67	3.61063	0.05389

The combined residual was used to calculate an F value of 7.28 ($F_{.01} = 4.95$) for antigen strain variation. The use of different antigen strains was a highly significant source of variation. The highest mean titer for antigen strain was produced with the S-91 strain which was isolated in this country. It seemed reasonable that antisera produced by local infecting strains would agglutinate to higher titers against an antigen strain which was isolated in the same general area. The lowest mean titer was found for the Johnson strain which was contrary to the results of the analysis of the survey data where it was found

to be the most sensitive strain. The higher laboratory means related to the use of the Johnson strain in the survey was probably due to the confounding of antigen strain with other factors.

An F value of 18.01 was calculated for reader variance ($F_{.01} = 7.04$) which was quite a bit higher than the 1 percent level. This highly significant degree of variation was not expected and it must be pointed out that the two readers compared were using two different microscopes which did not have the same power of magnification. However, this was the kind of variation which undoubtedly contributed greatly to the differences among laboratory means in the survey data. Further studies of this source of variation were made in the next experiment.

Effect of Test Reader and Microscope

The difference between the two microscopes in the previous experiment was suspected of contributing to the variation observed between the test readers. The Leitz Ortholux, magnification 150X, produced a vastly superior dark-field image than the Bausch and Lomb, magnification 100X. Although reader "HS" was not as accustomed to the Bausch and Lomb as reader "EC" an experiment was designed to compare the two readers on the two microscopes reading the same set of sera. Four sets of tests on fourteen sera were prepared employing a master set of fourfold dilutions, and one batch of antigen. The sera were double coded so that each reader was unaware of the identity or rank order of the sera. The experimental results (shown in Table 36) were of sufficient interest to present in their entirety.

The mean titers of the two readers were exactly the same when the

Table 36. Comparison of two test readers on two microscopes using fourteen sera

Sera	Microscope			
	Bausch and Lomb (100X)		Leitz (150X)	
	Reader		Reader	
	EC	HS	EC	HS
18	100	100	100	100
11	1,600	400	1,600	1,600
13	400	400	400	400
12	400	400	400	400
21	400	100	400	400
24	1,600	1,600	1,600	1,600
22	400	100	100	400
16	400	400	400	400
4	400	100	400	400
15	25,600	6,400	25,600	6,400
19	25	25	25	25
20	100	100	100	100
14	6,400	6,400	6,400	6,400
17	400	100	400	400
Mean titer	540	300	490	490

Leitz microscope was used. However, with the Bausch and Lomb microscope "EC" had a mean titer of 540 and "HS" a mean titer of 300. In Table 37 the complete statistical analysis was summarized.

Table 37. Analysis of variance of effects of serum, microscope, and reader

Source of variation	Degrees of freedom	Sum of squares	Mean square
Sera (S)	13	26.38697	2.02977
Microscopes (M)	1	0.10286	0.10286
Readers (R)	1	0.23143	0.23143
Interaction (SM)	13	0.25714	0.01978
Interaction (SR)	13	0.48857	0.03758
Interaction (MR)	1	0.23143	0.23143
Residual (SMR)	13	0.48857	0.03758

No interaction existed between sera and microscopes or sera and readers. However, the interaction of microscopes and readers was significant as indicated by an F of 6.16 ($F_{.05} = 4.67$, $F_{.01} = 9.07$). In the preceding experiment it was noted that antigen strain means of reader "EC" were higher than those of reader "HS" on all of the antigen strains. If these higher means were due to the use of the Bausch and Lomb microscope it would be expected that reader "HS" would have had a higher mean titer with it than with the Leitz. However, the contrary situation occurred. Reader "HS" expressed dissatisfaction with the Bausch and Lomb and felt some difficulty in determining end points. The F ratios for readers and microscopes were as follows:

$$F \text{ (Readers)} = 6.16,\ F_{.05} = 4.67,\ F_{.01} = 9.07$$

$$F \text{ (Microscopes)} = 2.74,\ F_{.05} = 4.67$$

From the above figures it would appear that significant variation occurred between readers in this experiment. The use of the two microscopes did not introduce significant variation as a whole. Individually, however, the better microscopic system (Leitz) permitted good replication of test reading between the two readers. Obviously, all of the reader variation occurred using the other microscope. The importance of a good dark-field system and adequate magnification of at least 150X when reading agglutination-lysis tests was emphasized by these findings.

SUMMARY

The replication studies on the Stoenner plate test and the agglutination-lysis test revealed a significant variation among repeat runs in the first test which was not found in the second. The Stoenner capillary tube test was performed on the test sera and the results were included in the survey. A sample correlation coefficient of 0.980 was calculated for the plate and capillary tube techniques as performed on the test sera. Sample correlation coefficients comparing the results of the Stoenner plate and capillary tube tests with those of the agglutination-lysis test were found to be 0.921 and 0.919, respectively.

Several experiments were performed on the agglutination-lysis test to determine what factors caused significant variation. The results of the experiments were discussed. The following factors were sources of significant variation: antigen density, serum dilution schemes, antigen strains, and test readers (personnel observing the end point titers). Some of the factors studied which were not found to be significant sources of variation were: antigen lots, readings by the same observer of tests prepared from the same dilution set, different dilution sets on the same lot of antigen, test incubation temperatures (23°, 30°, and 37°C.), and microscopes. Significant interaction was found between microscopes and test readers. The reason for this interaction was discussed.

From a statistical angle the comparison of factors should have been performed in one large experiment. However, the limitations of time and equipment made the development of such a procedure an impossibility.

PART III. RELATION OF SERUM ANTIBODY TITERS IN DAIRY CATTLE TO THE WHEY ANTIBODY TITER FOUND IN THE COMPOSITE HERD MILK SAMPLE

INTRODUCTION

The economic importance of leptospirosis to the livestock industry has been well established since it was first reported by Jungherr in 1944 (23). Considerable money is invested annually in the use of L. pomona bacterins for immunization against this disease. The alternative of control and eradication by sanitation and quarantine is regarded by many as impossible and, perhaps, unnecessary. The presence of leptospiral infection in the indigenous wildlife of the country is considered a source of infection which is impossible to control. However, other aspects of this disease are more encouraging. The short period of time during which an infected animal sheds leptospirae in the urine increases the effectiveness of suitable quarantine measures.

Needless to say, leptospirosis has reached the stage where some thought and effort should be made to develop a suitable control program. It would appear that the prevalence of L. pomona infection in cattle and swine has not been caused by wildlife reservoirs alone. Considerable infection between livestock must be conceded. Other serotypes are important in different countries of the world and their presence in the wild animal species of this country is a cause for concern. However, it has been stated that L. pomona was the agent in 98 percent of the outbreaks of leptospirosis in swine and cattle (24).

Control measures need not be test and slaughter programs but should be contrived so as to take advantage of the weak links in the chain of transmission. A control program for leptospirosis might be based on an economical screening test to locate the infection, quarantine or

surveilance of infected herds with concurrent vaccination and treatment until the carrier condition has passed, a periodic recheck of the serology of the infected herds, and special study and efforts in epizootic areas.

The value of the brucellosis milk ring test in locating Brucella infection suggested the development of a similar technique for the diagnosis of leptospirosis. Attempts were made to stain formalized suspensions of leptospira and prepare antigens which would produce a typical milk ring reaction. It was found that effective staining of the leptospirae with the technique used in preparing the brucellosis ring test antigen completely destroyed the antigenicity of the cells (25). Other stained leptospiral antigens also showed greatly reduced antigenicity. When these stained suspensions of leptospirae were placed in negative milk and the cream allowed to rise, excellent rings were produced. It was questioned whether the physical nature of the leptospirae would permit the cells to escape the sieving action of the fat globules even though no agglutination had taken place.

Attention was turned to the possibility that antibody levels in a herd milk sample, taken from either bulk tank or milk can, might be too low to permit detection. If this were true herds infected with leptospirosis could not be located in this manner. Since the agglutination-lysis test was the most sensitive serological technique it was decided to employ it on herd milk samples. The ability of this test to detect leptospiral antibody in bulk tank or milk can samples might lead to its successful application as a convenient screening test for the location of herds infected with leptospirosis. The project described here was

intended to determine the feasibility of this procedure and, if a measurable amount of antibody was present, to compare the titer of the herd milk sample with the serum titers found in the individual cattle.

LITERATURE REVIEW

The presence of specific antibody substances in milk has been recognized for some time. In 1893, the German chemists, Brieger and Ehrlich (26), succeeded in separating an "antitoxin" from the milk of a goat which had been immunized by repeated inoculations with the toxic products of the tetanus bacillus. A chemical process was used to extract a fraction from the milk which was four to six hundred times as effective in neutralizing the tetanus toxin as the milk itself.

In 1915, Seddon (27), in Australia, attempted to extend the application of the agglutination test for brucellosis to the milk of cattle but found the diluted milk unsuitable because of its physical properties. It was found that if the whey was separated from the milk a satisfactory test could be performed. The whey was prepared by mixing nine parts of milk with one part of a 10 percent aqueous solution of lactic acid. The coagulated milk was removed with filter paper. Serial dilutions of the whey were prepared in phenolized saline and an "emulsion" of Brucella cells was added. It was found that the agglutinins in the whey increased and decreased in the same manner as those in the blood serum.

Cooledge, in 1916, employed both milk and "rennet milk serum" as a means of indicating the presence of infection with Bacterium abortus (28). Complement-fixation and tube agglutination tests were utilized. It was found that the inoculation of a 48 hour culture into the milk cistern of a cow caused the appearance of agglutinins in the milk. The milk test was considered of diagnostic value since agglutinins

were always present in the milk when the organism was isolated.

The relation of the blood serum titer to the milk whey titer was further studied by Smith et al. (29). In cows inoculated subcutaneously or intravenously with killed suspensions of Brucella cells the ratio of antibody content in the milk to that in the blood was found to be 1 : 32 and 1 : 64. However, when the udder was invaded or made the site of multiplication by B. abortus then the whey agglutinins rose to a new ratio of 1 : 4, 1 : 2, and even 1 : 1.

In 1952, van der Hoeden (30) called attention to the large amounts of agglutinin present in the milk of cows infected with leptospirosis. Although the milk was occasionally negative in the early stages of the disease, positive agglutination tests from milk were always specific. A case of leptospirosis in a goat was described where the milk titer was 1 : 4,000 at the same time the blood titer was 1 : 20,000.

The application of a serological test to milk as a diagnostic technique for leptospirosis was described by van der Hoeden in 1955 (31). The clear whey obtained from a milk sample was diluted with a saline solution containing 0.4 percent formalin. Living leptospiral cultures were mixed with the whey dilutions and kept at room temperature until the following day. If examination under a microscope with dark-field illumination revealed "typical clumping" of the spirochetes the test was considered positive. The examination of blood and milk samples taken from 100 cattle infected with either L. grippotyphosa or L. canicola indicated the presence of a whey agglutinin titer approximately a tenfold dilution lower than the concomittant blood titer. Three cows with blood titers of 1 : 2,000, however, had negative whey titers.

The whey titer was considered more specific since heterologous antigens always gave negative results. The whey test was recommended for use where the collection of blood would meet with technical difficulties or where a screening test was desired. Negative results with the whey test were not considered conclusive.

Morse et al. (32) reported on the correlation between the milk whey and blood serum titers of infected cattle. The milk whey was obtained through the action of rennet on skimmed milk samples held at 4°C. for 18 hours. Whey agglutinins were found at levels from 1 : 10 to 1 : 10,000 in a herd of cattle where the onset of leptospirosis had occurred about two months previously. The whey titers seemed to be higher in cattle which had recently commenced lactation. Whey reactions were not found in cattle with blood titers of 1 : 10 or 1 : 100. The higher whey titers found in recently infected herds and the persistence of the whey titers for "many months following infection" were emphasized.

Fennestad and Borg-Petersen (33) studied whey titers in heifers artificially infected with different leptospiral serotypes. The colostral whey titer was found to be three to ten times higher than the serum titer but after 24 hours the whey titer to serum titer ratio had dropped to 1 : 10 or 1 : 100. Whey agglutinins could not be detected at the 1 : 30 dilution in these heifers 26 days after parturition in contrast to the finding of Morse et al. (32).

METHODS AND MATERIALS

Blood samples were obtained from the local brucellosis testing laboratory and carefully identified as to name and address of herd owner, name and address of the veterinarian submitting the samples, and location by county and township. Only complete tests of dairy herds from the 33 counties within a hundred mile radius of Ames were selected. The samples were tested with the Stoenner plate test (7) and herds showing positive reactions were retested with the agglutination-lysis test. The serum dilutions in the agglutination-lysis test were performed so as to produce final dilutions of 1 : 10, 1 : 100, 1 : 1,000, etc. Otherwise, the test was applied as described previously using the type strain L. pomona Pomona as the antigen culture. Negative herds as well as positive herds were visited in the field through preliminary arrangements with the veterinarians. Milk samples were obtained on the farm from the bulk storage tank or from the milk cans representing a single milking. Some quarter milk samples were also collected from individual cows. A herd history was procured from the herdsman or the veterinarian at the time of the visit.

The milk samples were collected in screw-capped vials and transported to the laboratory in a portable ice chest. After standing overnight at 4°C. the cream was removed by aspiration. Approximately 5 ml. of the skimmed milk was placed in a small test tube and 3 to 5 drops of a liquid rennet extract was added (Cheese Rennet, Chr. Hansons Laboratory, Inc., 9015 W. Maple Street, Milwaukee, Wisconsin). The tubes were agitated and placed in a water bath maintained at a

temperature of 37°C. for 2 to 4 hours. The clotted milk was gently loosened from the sides of the tube with a clean wood applicator stick and the whey was removed with a pipet after centrifugation. The whey samples were stored in the frozen state or tested immediately.

Agglutination-lysis tests on the whey were performed in the same manner as previously described except that a different dilution scheme was utilized. The lowest dilution examined was prepared by mixing equal amounts of whey and the antigen producing a final whey dilution of 1 : 2. Subsequent twofold dilutions were prepared from the whey so as to accurately determine the agglutinin content of the whey. The whey dilutions were, therefore, 1 : 2, 1 : 4, 1 : 8, and so on to the end point titer. A different dilution scheme was used on the quarter milk samples of 1 : 10, 1 : 40, 1 : 160, etc.

The antigen used in the Stoenner test was prepared commercially (Fort Dodge Laboratories, Inc., Serial Number 306124).

Composite herd milk samples from farms in the Ames area were examined in a preliminary survey through the courtesy of the Veterinary Hygiene Department, Iowa State University. No blood samples were obtained from these herds.

Since the purpose of the investigation was to determine the feasibility of a screening test employing a composite herd sample, the herds were not selected in a random manner. An effort was made to include in the survey all categories from negative herds to herds with strong serological indications and recent signs of leptospirosis infection.

RESULTS

There were two stumbling blocks expected in the task of detecting leptospiral agglutinins in composite herd milk samples. First, the level of antibody in the milk of a serologically positive cow would be diluted out by the milk of several negative cows. The bulk tank storage method would exert an even greater dilution effect than the use of milk cans. It would be necessary for the test to detect antibody levels at very low dilutions. Second, the physical nature of the whey might interfere with the dark-field examination of the agglutination-lysis tests performed on it. This difficulty would be increased when lower dilutions of whey were examined. A preliminary study was performed on herd milk samples from dairy farms in the vicinity of Ames. It was reasonably expected that some of the herds would be infected with leptospirosis. A total of thirty milk samples were tested from herds using the bulk tank storage method. The whey titers obtained from these samples are presented in Table 38. A second set of samples was procured four months later for comparison with the results of the first test. The herds were identified by the initials of the owner.

The whey dilutions were prepared as described commencing with a 1 : 2 dilution. The agglutination-lysis test on the 1 : 2 and 1 : 4 dilutions were occasionally difficult to read because of the light-scattering properties of substances in the whey. Otherwise, the tests were read in the usual manner. It helped to place rather thin drops on the slide and examine the edges of the drop when reading the tests under the dark-field microscope.

Table 38. Whey agglutination-lysis test results on dairy herds from the area around Ames, Iowa

Herd	Whey titer[a]	
	April	October
CDF	N	N
W & M	32	8
PT	N	N
CDT	16	8
AHD	N	N
HL	N	N
AS	N	2
RSS	N	N
KH	32	16
CD	N	4
DF	N	64
FF	2	2
BF	N	N
FLM	N	16
CR	N	N
EM	N	N
LCA	N	N
ON	N	N
CEW	4	N
FS	N	N
ORB	N	N
HF	2	N
MA	N	N
CM	N	N
DF	2	4
HO	N	N
AR	N	N
JB	2	N
LD	N	N
RC	N	N

[a]Twofold dilution scheme (1 : 2, 1 : 4, 1 : 8, etc.).

Three herds W & M, CDT, and KH showed fairly consistent titers at and above the 1 : 8 dilution on both tests. Out of a total of 30 herds, negative titers were found on both tests for 18 herds. Herds DF and FLM which were negative on the first test developed titers of 1 : 64 and 1 : 16, respectively, on the second test. Seven were classified as

positive in the 1 : 2 or 1 : 4 dilutions on either or both of the two tests.

The next logical step was to obtain a correlation between the blood serum titers and clincal signs of leptospirosis found in the individual cows with the whey titer detected in the herd milk sample. Table 39 summarizes this relationship with data obtained from seventeen diary herds. The herds are identified by a code number and listed in the order of ascending whey titers.

Herds 16 and 20 were considered negative controls for the investigation. However, herds 4 and 1 gave negative herd whey tests even though some cattle in these herds revealed serum titers. Herd 1 was extremely large and the milk from the 8 cattle possessing titers of $1:10^3$ was diluted considerably by the 117 negative cattle.

It was considered significant that herds 14 and 9 which gave a whey titer of 1:8 contained cattle with titers of $1:10^4$ and reported current signs of leptospirosis. Herd 16 contained 13 cattle all of which showed titers of at least 1-10. The absence of negative cattle in this herd accounted for the 1:16 whey titer without current signs of leptospirosis.

The whey titers of 1:256 for the bulk samples of herds 17 and 19 and the highest milk can whey titer of 1:512 from herd 18 were examples of what might be expected from herds where active leptospirosis infection was present. Since the whey obtained from the milk samples of herd 18 was of excellent quality a Stoenner plate test was performed on it. All three milk can whey samples from this herd reacted at the 1-10 dilution of the Stoenner test.

Herd 15 was of interest in that it showed a whey titer of 1:128

Table 39. Relation of composite milk sample whey titers to serum titers and histories of 17 dairy herds

Herd no.	Agglutination-lysis test (number of cattle)							Stoenner plate test (number of cattle)					History[a]	Whey AL tests (titer)	
	N	10	10^2	10^3	10^4	10^5	10^6	N	10	40	160	640		Bulk tank	Milk[b] can
16	18							18					N	N	
20	16							16					N	N	
4	8	7						7	7	1			+V		N
1	117	2	16	8				122	12	9			N	N	
14	9	3	7	3	2			18	3	1	2		++V	8	
9	6	12	3	5	1			No test					++V	8	
2		9	4					1	8	4			+V	16	
11	5	1	7	5	2			9	6	5			+V		16
12	1	1	6	4	1			6	2	5			N	16	
5	4	8	8	1				4	14	3			+V		32
7	7	4	3	2				No test					++V		32
8	2	6	5	4	3			No test					N	32	
3	8	2	8	4				7	7	6	1	1	++V		64
15	13			1	1			13	1			1	N		128
17	15	5	3	9				20		4	3	5	++V	256	
19			1	3	6	2	2		2	4	4	4	++	256	
18		1	2	6	6	2		1	3	5	4	4	N		512

[a]The herd history was coded as follows: N no signs of leptospirosis, + signs of leptospirosis at least one year previous to the collection of smaples, ++ current signs of leptospirosis, and V vaccination with L. pomona bacterin.

[b]Highest milk can sample titer.

although only two cattle revealed titers of $1:10^3$ and $1:10^4$ and the remaining thirteen cattle were negative. This herd was recently assembled accounting for the absence of signs of leptospirosis reported by the owner.

Following Stoenner's interpretation, herds 14, 3, 15, 17, 19 and 18 were considered "possibly infected" and revealed whey titers of from 1 : 8 to 1: 512. Two herds, 4 and 1, showing only titers of 1 : 40 and lower failed to react to the whey test. Four herds with maximum Stoenner titers of 1-40 produced whey titers of 1-16 and 1-32.

A limited investigation was made on the individual quarter titers developed by cattle which had blood agglutinin titers against L. pomona. The results are summarized in Table 40 with the cattle arranged in the order of ascending serum titers.

The most striking feature of the data was the variation in whey titer found for cows with the same serum titer. Cattle showing a serum titer of 1 : 10 produced whey agglutinin titers of from negative to 1 : 40. Likewise, cattle showing serum titers of 1 : 1000 revealed whey titers of N to 1 : 640. On the other hand, the similarity of quarter titers found for the same cow was also noteworthy. An F value of 1.70 ($F_{.05} = 2.78$, $F_{.01} = 4.16$) was calculated for the variation among quarters on the same cow. Data on the stage of lactation of each cow were not obtained.

Table 40. Agglutination-lysis test results on whey quarter titers and blood serum titers of 19 dairy cows

Cow number	Serum[a] titer	Quarter whey titer[b]			
		RF	RR	LR	LF
W22	10	N	N	N	N
0770	10	N	10	N	N
2878	10	N	N	10	10
9	10	10	10	10	10
0078	10	40	40	40	40
W62	100	N	N	N	N
W71	100	10	10	10	10
231010	100	10	10	10	10
5	100	10	40	10	10
1679	100	40	40	10	40
664206	100	40	40	40	40
8	100	40	160	160	160
4095	1000	N	N	N	N
664204	1000	10	10	10	10
W79	1000	10	10	10	10
8047	1000	40	40	40	40
4973	1000	40	40	40	40
6545	1000	160	160	160	40
8046	1000	160	640	160	160

[a]Tenfold dilution scheme ($10-10^6$).

[b]Fourfold dilution scheme (10-640).

DISCUSSION

The ability of the whey agglutination-lysis test to detect leptospiral antibodies in bulk tank herd milk samples was amply demonstrated by the results in Table 38. Unfortunately the blood serology and history of these herds were not available. Assuming a titer of 1-8 as indicating infection, five of the thirty herds may be classified as "possibly infected". An over-all infection rate of 4.9 percent for herds has been reported in Iowa (34). This value compares with the infection rate of these herds.

As mentioned, some difficulty was occasionally experienced in reading the tests in the 1 : 2 and 1 : 4 dilutions of whey. The scattering of light from materials in the suspension made it difficult to see the leptospirae. It was possible, however, to place a coverslip on the whey-antigen drop and examine under high power for leptospirae which had not been agglutinated. The careful preservation of the samples through cooling and the use of an active rennet aided in the production of an almost water clear whey sample.

The development of whey titers by herds DF and FLM (Table 38) on the second test which were negative on the first test would seem to indicate the onset of leptospirosis in these herds. The number of negative whey titers seems to indicate a reasonable degree of specificity for the test.

The foremost question to be answered from the data in Table 39 was what should be the minimum titer used to indicate herds suspected of infection with leptospirosis. Two herds showing current signs of

leptospirosis, agglutination-lysis titers of $1:10^4$, and Stoenner titers of 1:160 gave whey titers of 1:8. Considering the disease signs accompanied by recognized diagnostic titers in these herds it would appear that this should be the critical dilution. From a practical standpoint, a dilution of 1:8 or 1:10 could be prepared directly instead of stepwise as in this study and a single agglutination-lysis test performed for each herd. Selection of a minimum titer of 1:16 or even 1:32 would designate as positive some herds in which leptospirosis was no longer in the active phase. However, a margin of error must always be allowed and a screening test should be designed so that so-called "false positive" rather than "false negative" readings are generally made.

It was impossible to evaluate the effect vaccination might have on the whey titers since all the vaccination was performed on herds which reported present or past signs of leptospirosis. The absence of a positive history in herds 12, 8, and 18 must be considered in the light of the natural reluctance of some dairy herd owners to divulge information to representatives of government agencies. The owner of herd 12, however, kept swine in the dairy barn and lot. Abortions or other signs of leptospirosis were denied by the owner of herd 8, but he willingly supplied the information that his son's herd on an adjacent farm was infected. Herd 18 was pastured in a field with a common fence next to herd 19, which had severe signs of leptospirosis, and a small brook passed through the farm of herd 19 into the farm of herd 18.

The whey test on composite herd milk samples could be used to locate dairy herds infected with leptospirosis in the same manner as the brucellosis milk ring test. The technical difficulties of preparing

whey from the milk samples and setting up agglutination-lysis tests would be more than offset by the alternative problem of collecting and testing blood samples. The expense of finding an infected herd is calculated according to the number of clean herds which must be tested to locate it. If the desire should arise in the livestock industry for some sort of a control program for leptospirosis, whether by vaccination, surveillance, or quarantine, this test should prove a useful diagnostic tool in dairy farming areas. Further experimental work should be done involving a larger number of herds than were included in this study. The low level of the leptospiral agglutinins in the composite herd milk sample would appear to discourage the use of macroscopic tests with killed antigens to detect them.

The whey titer of the individual cow seems to vary considerably in relation to the serum titer. It would have been more informative if Table 40 had included the stage of lactation for each cow. According to Morse et al. (32) the agglutinins are highest in the first month of lactation and after the fourth month tend to drop off to 1:10 or negative. The absence of a significant difference between the quarter titers on the same cow was regarded as evidence that localized udder infection does not occur in leptospirosis. During the acute phase of the disease when the leptospiremia occurs there is a generalized antigenic stimulation of the animal body. The mammary gland shares in this effect equally in all four quarters. After the acute phase antibody production begins and detectable levels may be found in the milk. Since there is no significant difference between the quarter titers, it would appear that a chronic infection of the udder, as is seen in brucellosis, does

not occur. Infection of one quarter would tend to cause a higher antibody titer in the milk from it than from the other quarters.

The detection of dairy herds infected with leptospirosis would seem to be assisted by the efforts most dairy farmers make to keep their cows in different stages of lactation. An infected cow contributing milk reacting to a titer of 1:160 could have its milk diluted by a number of cows producing negative milk and still be detected in the composite sample.

SUMMARY

Consideration was given to means by which antibodies against leptospirae might be detected in the composite herd milk sample as obtained from the bulk tank or milk can. The agglutination-lysis test was used to successfully detect antibodies in the whey obtained from the herd sample. Blood samples were obtained from seventeen herds and examined with the agglutination-lysis test and the Stoenner plate test. The herds were visited, histories obtained, and a composite herd milk sample collected.

From a comparison of the data obtained, it was concluded that a whey titer of 1:8 in the composite herd sample was the critical titer to be used in screening for leptospirosis. Titers of 1:256 and 1:512 were detected in the milk of herds in which active leptospirosis infection was present.

Quarter milk samples were obtained from nineteen cows showing blood serum antibody titers. Statistical evaluation indicated that there was no significant difference between quarter whey titers of the same cow.

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APPENDIX

Code _____

Stoenner Test Questionnaire

1. When performing the screen test how do you measure the amount of serum used?

2. How do you calibrate the antigen dropped used to measure the antigen?

3. Describe method of incubating the test giving time and temperature of incubation, number of plate rotations before and after the incubation period, etc.

4. Describe technic of preparing serum dilutions for the end titer determinations.

5. How is the end titer of the serum determined? [50% end point, 100% end point, etc.]

6. Do you observe the test macroscopically or use some degree of magnification as a visual aid?

7. Are end titer determinations made on all serums reacting to the plate screen? Are these titers reported to the veterinarian?

Leptospirosis Unit
ADE Diagnostic Laboratory
Veterinary Quadrangle, I.S.C.
Ames, Iowa

Code No. _____

Agglutination Lysis Questionnaire

Please complete and return with test results.

1. Identify the strain of L. pomona antigen used in testing these samples and state where and when it was obtained. [Johnson, Pomona, etc.]

_____

_____

_____

2. Describe medium used to grow antigen and method of preparation.

_____

_____

_____

_____

3. How do you standardize your antigen? [age of culture, darkfield observation, counting methods, photoelectric technics, centrifugation time and speed used to throw down "breeding nests" and precipitates, etc.]

_____

_____

_____

_____

4. How is your glassware maintained? [cleaning methods, number of rinses, etc.]

_____

_____

_____

_____

5. Describe your serum dilution technic in detail giving the formula of the diluting fluid and the pipetting method.

6. How are the test dilutions incubated?

Hours _____ Temperature _____

Water bath _____ Air incubator _____

Other _____

7. Describe your method of reading the test giving brand name of microscope, magnification, type of darkfield [oil, dry, or modified Abbe Condenser] and basis for approximating end points [50% agglutination lysis, etc.]

8. Comments [feel free]